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THE UNIVERSITY OF ALBERTA

Purification, chemical characterization, host-specificity, bioassay, mode of action, and herbicidal use of the toxin produced by *Alternaria brassicae*

by

Piara Singh Bains

(C)

A THESIS

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Supervisor

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External Examiner

Date...March 31, 1989.....

To

my wife Gogi, for being so special, and to

the memory of my late father, Sarbdial S. Bains.

Abstract

A chromatographic procedure was developed to purify the toxin produced in culture by *Alternaria brassicae*. The procedure included gel filtration, adsorption on charcoal, silicic acid chromatography and high pressure liquid chromatography. Negative ninhydrin reaction of the toxin and the presence of amino acids in the toxin hydrolysate indicated that the toxin is a cyclic peptide. Molecular weight, ninhydrin reaction, amino acid composition, proton nuclear magnetic resonance spectrum, HPLC retention time, and symptomatology and activity of the toxin on hosts and nonhosts of *A. brassicae* were identical with those of destruxin B. The degrees of toxin sensitivity of all plants tested were correlated with their degrees of susceptibility to *A. brassicae*, suggesting that the activity of *A. brassicae*-toxin is host-specific. This is the first report on the host-specific nature of a toxin produced by *A. brassicae*.

Comparison of four toxin bioassay methods indicated that the pollen germination bioassay was the most sensitive, quantitative, fast, and simple method for destruxin B. Significant inhibition of both germination and pollen tube growth of *Brassica campestris* var. *yellow sarson* pollen was observed after only 30 min of incubation with 2.5 μg/ml destruxin B, and complete inhibition of germination was observed at 7.5 μg/ml of the toxin.

Absence of rapid electrolyte leakage suggested that the plasma membrane may not be the initial site of destruxin B action. Non-sensitivity of protoplasts from the susceptible host and sensitivity of leaves and pollen from the same host appear to indicate that the target site of destruxin B is in the region of the cell wall and/or the periplasmic space.

Differential germination response of pollen from susceptible and resistant plants indicated that *in vitro* microspore culture in the presence of destruxin B is likely to be an excellent system for *in vitro* selection for *A. brassicae* resistance.

Destruxin B severely affected all cruciferous weeds tested except peppergrass and shepherd's purse. Stinkweed and wild mustard developed severe chlorosis and necrosis that often resulted in the death of the plant. However, there was no affect of destruxin B on

barley, oats, rye, and wheat. These results suggested that destruxin B can be used to control some cruciferous weeds in cereals.

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I. General Introduction

A. Preface

Rapeseed is one of the more important oilseed crops in the world, and has been challenging soybean for first place in the world oilseed trade (Weiss, 1983). Rapeseed is grown both in tropical and temperate zones. The principal rapeseed growing countries in the world are: Canada in North America; China, India, and Pakistan in Asia; and France, Germany, Poland, and Sweden in Europe (Kolte, 1985). *Brassica campestris* L. and *B. napus* L. are the two oil producing species of rapeseed. Production of rapeseed has been increasing since 1960, and in 1980, the estimated total world production was around 12 million tons (Weiss, 1983). Canada is the second largest producer in the world, and the Canadian prairie provinces produced 3.1 million tons out of a total of 3.2 million tons produced in Canada in 1984 (Veeman, 1985). Increased importance of rapeseed on the world scene can be attributed mainly to the development of low erucic acid and glucosinolate varieties, called canola, the trademark held by the Canola Council of Canada. Low erucic acid oil is considered safe for human consumption, and low glucosinolate content in the meal made it acceptable for feed stock. Tower and Candle, the first two canola cultivars of *B. napus* and *B. campestris*, respectively, were developed in Canada, and subsequently many other canola cultivars with better yields and/or some other special traits were released (Kondra, 1985). Areas covered by these varieties has been increasing since 1970, and by 1983 the conversion into canola-types was essentially complete. Canada is the second largest producer and the largest exporter of rapeseed in the world, therefore, it is very important to the Canadian agricultural economy (Kondra, 1985; Veeman, 1985).

There are many diseases of rapeseed (Kolte, 1985; Petrie, 1975; Tewari, 1985). Blackspot caused by *Alternaria* spp. is an important disease of rapeseed in western Canada (Petrie, 1975; Tewari, 1985; Tewari and Conn, 1988), and around the world (Kolte, 1985; Weiss, 1983). The disease is generally caused by any of the three *Alternaria* spp., namely *A. brassicae* (Berk.) Sacc., *A. brassicicola* (Schw.) Wiltshire, and *A. raphani* Groves &

Skolko. Disease causing strains of *A. alternata* (Fr.) Keissler are also reported (Vaartnou and Tewari, 1972). Due to its severity the disease caused by *A. brassicae* is of high economic importance (Kolte, 1985). *Alternaria brassicicola* and *A. raphani* are of minor importance in the Canadian prairies (Tewari, 1985). *Alternaria brassicae* infects all aerial parts and reduces yield significantly (Degenhardt *et al.*, 1974; Kolte, 1985; Tewari and Conn, 1988).

There is little information available on the mechanism of pathogenesis by *A. brassicae*. This pathogen penetrates plant tissues either directly or through stomata (Tsuneda and Skoropad, 1978) and has an initial subcuticular phase (Tewari, 1986). There are two earlier reports suggesting that a toxin may be involved in disease development. Crude culture filtrates of *A. brassicae* caused toxic effects on *B. campestris* var. *yellow sarson* seedlings (Husain and Thakur, 1966), and two groups of compounds isolated from culture filtrates of the fungus caused disease symptoms in rapeseed leaves (Degenhardt, 1978). The toxin produced by *A. brassicae* was identified as destruxin B (Ayer and Peña-Rodriguez, 1987). Hormonal disturbance may also play a role in the pathogenesis by *A. brassicae*, as abscisic acid is known to be produced by this pathogen (Dahiya *et al.*, 1988). Resistance in crucifers to *A. brassicae* is related to the amount of epicuticular wax (Tewari and Skoropad, 1976; Skoropad and Tewari, 1977; Conn, 1986) and elicitation of phytoalexins (Conn *et al.*, 1988).

B. Host-Specific and Nonhost-specific Toxins

Toxins produced by plant pathogenic microbes are low molecular weight non-antigenic compounds that cause obvious damage to plants and are involved in disease development (Durbin, 1983; Scheffer and Livingston, 1984). The toxins are generally classified as host-specific or nonhost-specific. Host-specific toxins are toxic only to the hosts of the producing pathogens, whereas, nonhost-specific toxins are toxic to many plants, whether hosts or nonhosts of the producing pathogens (Pringle and Scheffer, 1964). Host-specific toxins are conceptually important to the field of plant pathology because they

can explain the restricted host range of pathogens, whereas, nonhost-specific toxins seem to affect a factor common to all or many plants and act after the compatibility reaction has already taken place (Mitchell, 1984; Walton and Earle, 1984). Toxins have also been referred to as pathogenicity or virulence factors (Yoder, 1980). Most host-specific toxins are pathogenicity factors meaning that they are required by the organism to induce disease, whereas, nonhost-specific toxins are virulence factors indicating that they are not required by the organism for pathogenicity but that they contribute to the virulence of the organism (Scheffer, 1983). In the last decade many review articles (Daly, 1982, 1987; Daly and Knoche, 1982; Durbin, 1981; Janardhanan and Husain, 1983; Kohmoto *et al.*, 1987; Marré, 1980; Nishimura, 1987; Nishimura and Kohmoto, 1983a, 1983b; Nishimura *et al.*, 1979, 1982; Scheffer and Livingston, 1984; Strobel, 1982; Yoder, 1980), and books (Daly and Deverall, 1983; Durbin, 1981) have been written on toxins in plant diseases. This review will concentrate on presenting the available information on phytotoxins, more specifically on those that are host-specific and are produced by phytopathogenic fungi (Table I-1).

C. Molecular Disease Determinants

Presently, one of the thrusts in plant pathological research is towards the understanding of molecular determinants and their specific roles in host-parasite interactions (Nishimura, 1987). There is an agreement among researchers that host-parasite interactions require complementary chemical recognition (Daly, 1984). Scheffer (1983) listed a number of pathogen-produced known or hypothetical disease determinants including toxins, enzymes, hormone-like or anti-hormonal compounds, tumour-inducing DNA, pathogen surface carbohydrates, and products that interfere with the movement of water, nutrients and metabolites. Out of these, tumour-inducing DNA from *Agrobacterium tumefaciens* (Sm. & Towns.) Conn (Kahl and Schell, 1982) and several host-specific toxins are now

Table I-1. Host-specific toxins produced by phytopathogenic fungi.

Toxin	Producing Fungus	Plant Affected
AAL	*Alternaria alternata* f. sp. *lycopersici*	Tomato
ACRL	*A. citri* affecting rough lemon (now known as *A. alternata* rough lemon pathotype)	Rough lemon
ACTG	*A. citri* tangerine pathotype (now known as *A. alternata* tangerine pathotype)	Dancy tangerine
AF	*A. alternata* strawberry pathotype	Strawberry
AK	*A. alternata* Japanese pear pathotype	Japanese pear
AM	*A. alternata* apple pathotype	Apple
HC	*Helminthosporium carbonum*	Maize
HMT	*H. maydis* race T	Maize
HO	*H. oryzae*	Rice
HS	*H. sacchari*	Sugarcane
HV	*H. victoriae*	Oats
PC	*Periconia circinata*	Sorghum
PM	*Phyllosticta maydis*	Maize

Modified from Scheffer and Livingston (1984).

known to be determinants of plant disease development (Daly, 1984; Scheffer, 1983). Host-specific toxins are perfect candidates to understand biochemical bases of disease development, disease resistance, and host-specificity (Nishimura and Kohmoto, 1983a; Scheffer and Livingston, 1984; Wheeler, 1976; Wolpert and Dunkle, 1980; Yoder, 1980).

D. Role of Toxins in Disease Development

Many researchers have suggested various guidelines to evaluate the role of toxins in disease development. These include the development of disease symptoms upon toxin application to the healthy plant tissue, isolation of the toxin from diseased tissue, correlation of pathogen specificity with the toxin specificity, correlation of virulence of a pathogen with its ability to produce toxin (Yoder, 1980, 1981), and release of toxin from germinating spores (Nishimura and Kohmoto, 1983a, 1983b). Yoder (1980) discussed the strengths and weaknesses of these points and suggested that none of these criteria alone is adequate to prove the role of toxin in disease development. The degree of confidence increases when combinations of these criteria are used. There seems to be a consensus among researchers that the most compelling evidence for the role of toxin in pathogenesis comes from their selective toxicity on the host plants of the pathogen involved (Wheeler, 1981).

Nishimura and Scheffer (1965) reported that the toxins released by the germinating spores of pathogenic, but not nonpathogenic, isolates of *Helminthosporium victoriae* Meehan & Murphy are important in disease development. Since spores of both virulent and avirulent isolates showed no difference in germination and in the initial stages of penetration (Paddock, 1953), it was concluded that a toxin was the key factor in disease development. Later, Yoder and Scheffer (1969) demonstrated that when *H. victoriae* toxin (HV-toxin) was added to an infection drop of the nonpathogenic isolate, it facilitated infection in the susceptible tissue. They concluded that the toxin was required for initial colonization of the susceptible tissue, and was responsible for initial changes in host cell physiology. Similar results were obtained for AK-toxin produced by *A. kikuchiana* Tanaka

(now known as *A. alternata* (Fr.) Keissler pear pathotype) (Otani *et al.*, 1975). It was suggested that the toxin is a pathogenicity factor, and that electrolyte leakage is the initial physiological change. Since then, similar approaches have been used to demonstrate that AAL-toxin produced by *A. alternata* f.sp. *lycopersici* (Kohmoto *et al.*, 1982b), AF-toxin produced by *A. alternata* strawberry pathotype (Yamamoto *et al.*, 1984), AM-toxin produced by *A. alternata* apple pathotype (Kohmoto *et al.*, 1976), HC-toxin produced by *H. carbonum* Ullstrup (Comstock and Scheffer, 1973), and *H. oryzae* Breda de Haan toxin (Vidhyasekaran *et al.*, 1986) are pathogenicity factors. Results from all of these studies confirmed that the presence of a toxin at the site of contact of host and pathogen is important for the recognition phenomenon as well as for the successful infection of the host tissue (Nishimura and Kohmoto, 1983a). Role of a toxin in disease development can also be monitored by inhibiting production of the toxin during spore germination (Nishimura and Kohmoto, 1983b). Heat or cerulenin-treated spores of *A. alternata* Japanese pear pathotype, *A. alternata* apple pathotype, and *A. alternata* strawberry pathotype lost their pathogenicity and ability to produce host-specific toxins, but their germination, elongation of the germ tube, and appressorium formation were not significantly inhibited (Tsuge and Nishimura, 1984; Tsuge *et al.*, 1985). The results suggested that the pathogens depended upon their toxins for pathogenicity.

E. Degree of Specificity of Host-Specific Toxins

Host-specific toxins show various degrees of specificities. HV-toxin is highly specific it only affects the varieties of oats containing the 'Victoria' gene (Meehan and Murphy, 1947; Scheffer, 1976), whereas, *Hypoxylon mammatum* (Wahl.) Miller toxin is species-specific (Stermer *et al.*, 1984). In a few cases, however, slight discrepancies do occur between the host range of the fungus and the plants affected by its toxin. One of the two distinct pathotypes of *A. citri* Ellis & Pierce affecting rough lemon (now known as *A. alternata* rough lemon pathotype) produces toxins (ACRL-toxins) of high specificity. However, there is one notable exception; Cleopatra mandarin, which is resistant to the

fungus is sensitive to the toxin (Gardner *et al.*, 1985a). Host-specific ACTG-toxin produced by *A. citri* tangerine pathotype (now known as *A. alternata* tangerine pathotype) (Kohmoto *et al.*, 1979) affects nonhost plants to various degrees, indicating that the toxin is semiselective or has different degrees of selectivities (Gardner *et al.*, 1986). Sensitivity of some sugarcane clones to *H. sacchari* (Van Breda de Haan) Butler toxin (HS-toxin) is not correlated with their susceptibility to the fungus (Scheffer and Livingston, 1980), suggesting that the toxin may be one of several factors responsible for pathogenicity. Similar results have also been obtained for the clones of *P. tremuloides* and *H. mammatum* toxin (Stermer *et al.*, 1984). Payne and Yoder (1978) indicated that the levels of susceptibility and sensitivity of corn to *H. maydis* Nisikado & Miyake race T and its toxin (HMT-toxin) are influenced by the nuclear genome. Furthermore, differences in sensitivity to the toxin did not correlate with differences in susceptibility to the fungus. The results indicated that the toxin is not required for a compatible host-pathogen relationship but contributed to its virulence (Payne and Yoder, 1978; Yoder, 1981). Hawes and Wheeler (1984) and Sugawara *et al.* (1987) also suggested that factors other than the toxin may be involved in pathogenicity.

F. Isolation and Purification of Toxins

Availability of pure toxins is a prerequisite for their use in studies aimed at understanding their role in disease resistance, susceptibility and host-specificity. Many earlier studies describing roles of toxins in disease development were not taken seriously because they were conducted using toxins of uncertain purity (Daly, 1987). Although pure toxins should be used for all the experiments, partially purified toxins can be used for preliminary work (Shaw, 1981). Some excellent work has been done using partially purified toxins (Walton and Earle, 1984b). However, critical work, such as mode of action studies, should only be conducted using a highly purified toxin (Daly, 1981), because impurities may cause nonselective activity, leading to erroneous interpretations (Arias *et al.*, 1983; Macko *et al.*, 1981; Shaw, 1981).

Fungal Culture

Fungi for toxin production can be grown either in liquid culture or on a solid medium (Shaw, 1981). Almost always, liquid cultures are preferred because fungi usually grow slowly on solid media, toxins may be trapped or adsorbed by agar or silica particles, and agar impurities may interfere with the isolation of the toxin (Shaw, 1981). Liquid cultures of fungi could be still or agitated. In the majority of cases, still cultures of fungi have been used (Clouse *et al.*, 1985; Kono *et al.*, 1986b; Livingston and Scheffer, 1981; Nakashima *et al.*, 1985; Ueno *et al.*, 1975a; Walton and Earle, 1984b; Walton *et al.*, 1982; Wolpert and Dunkle, 1980), but in some studies shake cultures have also been used (Van Etten and Daly, 1980). The use of shake cultures reduces the time for maximum toxin production and rapidly depletes the nutrients of the medium, thereby making the purification process slightly easier, but it causes the mycelium to clump together and creates an unfavourable interface for exchange with the medium (Daly and Knoche, 1982; Van Etten and Daly, 1980).

Fungi for toxin production have been cultured on chemically defined as well as complex media. Chemically defined media that have been used include Czapek medium (Gardner *et al.*, 1985a; Karr *et al.*, 1974; Kohmoto *et al.*, 1979; Kono *et al.*, 1986b), and modified Richard's medium (Kohmoto *et al.*, 1984; Nakashima *et al.*, 1985; Ueno *et al.*, 1975a). In many instances chemically defined medium was supplemented with yeast extract (Briggs *et al.*, 1984; Clouse *et al.*, 1985; Dunkle and Wolpert, 1981; Kohmoto *et al.*, 1982b; Livingston and Scheffer, 1981, 1983; Wolpert and Dunkle, 1980). *Helminthosporium victoriae* is routinely cultured on a medium containing host plant material such as oat flakes or oatmeal (Kono *et al.*, 1986c; Mayama *et al.*, 1986; Walton and Earle, 1984a; Wheeler and Gantz, 1981). Fungi have also been grown on very complex media such as potato dextrose broth (Gilchrist and Grogan, 1976), liquid V8 juice medium (Ayer and Peña-Rodriguez, 1987; Degenhardt, 1978). Culture of fungi for toxin production is greatly influenced by the environment and nutritional factors. Shaw (1981) has discussed this aspect in great detail.

Toxin Isolation

After the fungus has been cultured for the desired length of time the toxin can usually be isolated from crude culture filtrates. The toxin is isolated using some basic physical and chemical separation methods, and then further purified using more sophisticated techniques. Isolation of toxins generally involves a combination of some of the following methods: filtration, concentration, alcohol precipitation and solvent extraction (Walton and Earle, 1984b). In a few instances, chromatographic materials have also been used to adsorb the toxin in the early isolation steps. Activated charcoal was used to isolate toxins from culture filtrates of *A. alternata* tomato pathotype (Kohmoto *et al.*, 1982b), *H. maydis* race T (Kono and Daly, 1979), *H. mammatum* (Stermer *et al.*, 1984), *H. oryzae* (Vidhyasekeran *et al.*, 1986), *H. sacchari* (Livingston and Scheffer, 1983), *H. victoriae* (Kono *et al.*, 1986c), *Periconia circinata* (Mangin) Sacc. (Dunkle and Wolpert, 1981; Wolpert and Dunkle, 1980), and *Phyllosticta maydis* Arny & Nelson (Danko *et al.*, 1984; Kono *et al.*, 1983). On the other hand, Amberlite XAD-7 was included in the isolation procedures for toxins from culture filtrates of *A. alternata* tangerine pathotype (Kono *et al.*, 1986b), *A. alternata* rough lemon pathotype (Gardner *et al.*, 1985a, 1985b), and *H. victoriae* (Mayama *et al.*, 1986) and XAD-2 in the isolation procedure of toxin from culture filtrates of *A. alternata* Japanese pear pathotype (Nakashima *et al.*, 1982, 1985). A C_{18} reverse phase trap column was used to adsorb toxins from the culture filtrates of *A. alternata* tomato pathotype (Clouse *et al.*, 1985; Siler and Gilchrist, 1983). The column improved a labourious and time consuming scheme described earlier (Siler and Gilchrist, 1982).

Toxin Purification

For purification, chromatography is almost always performed. Chromatographic techniques separate molecules on the basis of their chemical characteristics such as size and ionizable groups. The most commonly used chromatographic techniques include gel filtration, thin-layer chromatography (TLC), adsorption chromatography, ion-exchange

chromatography, and high-pressure liquid chromatography (HPLC). Gel filtration is not normally suitable for small molecular weight toxins. In spite of low capacity, TLC has better reproducibility than adsorption chromatography but both have moderate resolution and are inexpensive and relatively easy to use (Walton and Earle, 1984b). Most toxin purification schemes include different chromatographic techniques in various combinations.

HV-toxin was purified by cation exchange chromatography using SP-Sephadex C25 and HPLC (Briggs *et al.*, 1984); by adsorption chromatography on alumina column, silica gel TLC, and HPLC (Walton and Earle, 1984a; Mayama *et al.*, 1986); and by column chromatography using Sephadex LH-20, followed by preparative silica gel TLC (Kono *et al.*, 1986c). HC-toxin was purified using preparative silica gel TLC and HPLC (Ciuffetti *et al.*, 1983), and by gel filtration on Sephadex G-15 and HPLC (Walton *et al.*, 1982). Karr *et al.* (1974) used gel filtration on Bio Gel P-2, paper chromatography, and TLC on Aborosil 3 plates to purify HMT-toxins. Livingston and Scheffer (1981) purified HS-toxin by Sephadex LH-20 gel filtration chromatography, Sephadex QAE anion exchange chromatography, and silica gel TLC. However, later they modified the procedure by replacing TLC with flash chromatography using Whatman LPS-2 and added reverse phase HPLC as the final step (Livingston and Scheffer, 1984a). Macko *et al.* (1981) purified three HS-toxin isomers by adsorption chromatography on silicic acid, droplet countercurrent chromatography, and HPLC. *Helminthosporium oryzae* toxin was isolated by adsorption chromatography on alumina, gel filtration with Sephadex LH-20, and TLC on silica gel 60 sheets (Vidhyasekaran *et al.*, 1986). The host-specific AM-toxins I and II were purified by adsorption column chromatography using silicic acid and silica gel TLC (Ueno *et al.*, 1975a; 1975b). Nakashima *et al.* (1982) purified two host-specific metabolites from the culture filtrates of *A. kikuchiana* (now known as *A. alternata* Japanese pear pathotype) by adsorption column chromatography using silica gel, droplet countercurrent chromatography and gel filtration by LH-20. However, later the method was modified by replacing gel filtration by another silica gel column (Nakashima *et al.*, 1985). Maekawa *et al.* (1984) purified three host-specific toxins, AF-toxins I, II and III, from culture filtrates of *A.*

alternata strawberry pathotype by different chromatographic techniques including adsorption chromatography using Sephadex LH-20, droplet countercurrent chromatography and silica gel TLC, however, this method was later modified to include HPLC as the final purification step (Namiki *et al.*, 1986b). The method used for isolation and purification of AAL-toxins included butanol extraction, gel filtration, and TLC (Siler and Gilchrist, 1982). Further improvement was made by replacing butanol extraction and gel filtration with a C_{18} reverse phase trap column and by addition of HPLC as the final purification step (Clouse *et al.*, 1985). Host-specific ACRL-toxins were purified by adsorption column chromatography using silicic acid, silica gel TLC, and HPLC (Gardner *et al.*, 1985a, 1985b). Ayer and Peña-Rodriguez (1987) developed a scheme to isolate destruxin B from crude culture broth extract of *A. brassicae*. Water/methanol (9:1) suspended extract was partitioned successively against hexane, ethyl acetate, and n-butanol. The medium polarity fraction showed the strongest biological activity, and destruxin B was isolated by dry flash column chromatography using silica gel 60. The purification procedure for *H. mammatum* toxin included Bio Gel P-2 gel filtration, silicic acid adsorption chromatography, and HPLC (Stermer *et al.*, 1984). The purification scheme for *Periconia circinata* (Margin) Sacc. toxin (PC-toxin) included QAE-Sephadex ion exchange chromatography, thin-layer electrophoresis using silica gel plates, TLC on polyamide sheets, and HPLC (Dunkle and Wolpert, 1981; Wolpert and Dunkle, 1980). The examples given above suggest clearly that HPLC is a method of choice for the final purification step. It has excellent resolution and reproducibility (Walton and Earle, 1984b), but only a small quantity of toxin can be purified.

G. Structure of Toxins

Knowledge of the structure of host-specific toxin, although not easily achievable, is a key prerequisite for toxin studies undertaken to understand the molecular basis of the plant disease (Gilchrist, 1983; Macko, 1983). Earlier studies were unable to provide such an information because they were conducted using impure toxins of unknown structure

(Macko, 1983). However, recent developments in purification procedures and analytical technology have helped to elucidate the structures of many host-specific toxins (Nishimura, 1987). They include high resolution mass spectometry (HR-MS), fast atomic bombardment mass spectrometry (FAB-MS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography - mass spectroscopy (GC-MS). Toxins whose structures have been elucidated using these techniques are listed in Table I-2.

The structure of destruxin B is shown in Fig. I-1. Crystallized destruxin B is in the form of white needles with melting point 225-227° and infra red spectrum ($CHCl_3$ cast) 3360, 3290, 1730, 1670, 1630, 1515 /cm (Ayer and Peña-Rodriguez, 1987). The ^{1}H NMR spectrum of this compound is shown in Fig. II-2.

H. Toxin Bioassay

Bioassay is required for every facet of toxin research. It cannot be replaced by any other method to define a toxin and describe its role in disease development (Yoder, 1981). A bioassay should be sensitive, quantitative, reproducible, and easy to perform. The choice of any bioassay depends upon the nature of the toxin, the nature of the plant, and the purpose of the experiment. Bioassays are used to detect new toxins, to purify toxins, to determine the role of toxins in disease development, to elucidate the mode of action of toxins, and to screen for disease resistance (Yoder, 1981).

For toxin detection and purification, a very sensitive bioassay is required, otherwise some toxins may go undetected and get discarded during the purification procedure (Yoder, 1981). The most commonly used bioassay techniques for toxin detection, toxin purification, and understanding the role of toxin in disease development include detached leaf bioassay, primary root growth bioassay, and electrolyte leakage bioassay (Table I-3).

Table I-2. Chemical types of toxins produced by phytopathogenic fungi.

Toxin	Chemical type	Reference
AAL	Ester of propanetricarboxylic acid	Bottini and Gilchrist, 1981
ACRL	Acetogenin	Gardner *et al.*, 1985b; Kono *et al.* 1985a, 1986b
AF	Acetogenin	Nakatsuka *et al.*, 1985 as referred by Nishimura, 1987
AK	Ester of epoxydecatrienoic acid	Nakashima *et al.*, 1982, 1985
AM	Cyclodepsipeptide	Okuno *et al.*, 1974; Ueno *et al.*, 1975b
Destruxin B	Cyclodepsipeptide	Ayer and Peña-Rodriguez, 1987
HC	Cyclic tetrapeptide	Gross *et al.*, 1982; Liesch *et al.*, 1982; Walton *et al.*, 1982
HMT	Linear polyketols with 35-40 carbon atoms	Kono and Daly, 1979; Kono *et al.*, 1980, 1985b
HS	Sesquiterpene glycoside	Livingston and Scheffer, 1981; Macko *et al.*, 1981

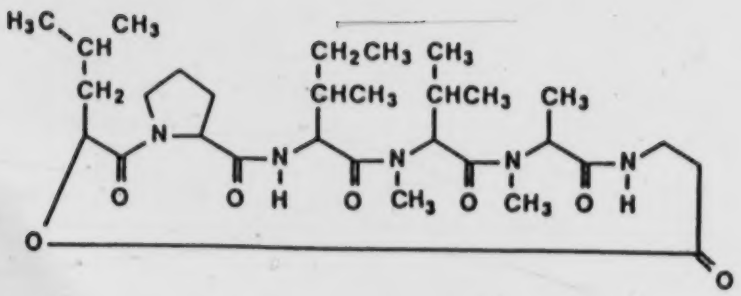

Fig. I-1. Chemical structure of destruxin B.

Adapted from Ayer *et al.*, 1987

Table I-3. Bioassays used for various host-specific toxins produced by phytopathogenic fungi.

Bioassay	Toxin	Reference
Detached leaf	AAL	Clouse *et al.*, 1985; Gilchrist and Grogan, 1976
	ACRL	Gardner *et al.*, 1985a, 1986
	AF	Maekawa *et al.*, 1984; Namiki *et al.*, 1986b; Yamamoto *et al.*, 1985
	AK	Nakashima *et al.*, 1982, 1985; Otani *et al.*, 1985a
	AM	Kohmoto *et al.*, 1976; Ueno *et al.*, 1975a
	Destruxin B	Ayer and Peña-Rodriguez, 1987
	HMT	Karr *et al.*, 1974; Kono and Daly, 1979
	HS	Lesney *et al.*, 1982; Steiner and Byther, 1971
Primary root growth	HC	Kim and Knoche, 1987
	HMT	Payne and Yoder, 1978; Karr *et al.*, 1974
	HV	Mayama *et al.*, 1986; Van Etten and Daly, 1980; Walton and Earle, 1984a; Wheeler and Gantz, 1981
	PC	Wolpert and Dunkle, 1980
Pollen germination	HMT	Laughnan and Gabay, 1973
Isolated cell viability	HMT	Hawes 1983b; Hawes and Wheeler, 1984
	HV	Hawes and Wheeler., 1982
Isolated protoplast viability	HMT	Aldrich *et al.*, 1977; Earle *et al.*, 1978; Gregory *et al.*, 1979; Pelcher *et al.*, 1975; Walton *et al.*, 1980; York *et al.*, 1980

Table I-3. (continued)

Bioassay	Toxin	Reference
	HV	Briggs *et al.*, 1984; Hawes, 1983a
Isolated mitochondria	HMT	Gregory *et al.*, 1980; Sugawara *et al.*, 1987; York *et al.*, 1980
Electrolyte leakage	ACRL	Kohmoto *et al.*, 1979
	AF	Yamamoto *et al.*, 1984
	AK	Otani *et al.*, 1975, 1985a
	HMT	Halloin *et al.*, 1973; Payne *et al.*, 1980b
	HS	Larkin and Scowcroft, 1981; Livingston and Scheffer, 1981, 1984a; Scheffer and Livingston, 1980
	HV	Damann *et al.*, 1974; Yoder and Scheffer, 1969; Samaddar and Scheffer, 1971
Inhibition of dark Co_2 fixation	HMT	Bervillé and Paillard, 1984; Bhullar *et al.*, 1975; Payne and Yoder 1978; Sugawara *et al.*, 1987; Yoder *et al.*, 1977
	HV	Kono *et al.*, 1986c
	PM	Danko *et al.*, 1984; Suzuki *et al.*, 1985
Inhibition of incorporation of amino acids	ACRL	Gardner *et al.*, 1985a, 1986; Kono *et al.*, 1985a
	ACTG	Gardner *et al.*, 1986
Stimulation of NADH oxidation and inhibition of malate oxidation	HMT	Suzuki *et al.*, 1983
	PM	Suzuki *et al.*, 1985, 1987

Bhullar *et al.* (1975) indicated that the electrolyte leakage bioassay is less sensitive than the root growth bioassay which is tedious, time consuming, and less reliable due to variation in seed germination. They developed the inhibition of dark fixation of CO_2 bioassay for HMT-toxin and suggested that it is rapid and reliable. A comparison of different bioassays for their simplicity and sensitivity to HMT-toxin indicated that dark CO_2 fixation was technically difficult to perform but was the most sensitive. The root growth bioassay was easy to perform and was of intermediate sensitivity, and the leaf lesion bioassay was the least sensitive but the easiest to perform (Yoder *et al.*, 1977). However, Payne *et al.* (1980a) reported that dark CO_2 fixation, ion balance, coleoptile elongation, and mitochondrial oxidation bioassays showed similar sensitivities to HMT-toxin. Scheffer and Livingston (1980) also observed that the leaf bioassay was not sensitive enough to differentiate sensitivity differences of sugarcane clones to HS-toxin, whereas the electrolyte leakage assay was sensitive enough to do so. The protoplast bioassay is sensitive and widely applicable (Walton and Earle, 1984b; Earle *et al.*, 1978; Briggs *et al.*, 1984). Root-cap-cell bioassay also has the characteristics of a good bioassay technique (Hawes, 1983a; 1983b; Hawes and Wheeler, 1982).

Erroneous results can be obtained if the bioassay used is not sensitive enough. HMT-toxin was earlier reported to be a pathogenicity factor (Turner and Martinson, 1972; Watrud *et al.*, 1975). However, with the help of more sensitive and quantitative bioassays such as seedling root growth and dark CO_2 fixation (Yoder *et al.*, 1977), it was discovered that the toxin is not a pathogenicity factor but a virulence factor (Payne and Yoder, 1978). Depending upon the bioassays used, nonhost plants of *A. alternata* tangerine pathotype showed various degrees of sensitivity to its toxin (Gardner *et al.*, 1986). Bioassays based on incorporation of proline into protein and loss of electrolytes showed a low level of selectivity, whereas leaf puncture and shoot cutting uptake bioassays indicated a high level of selectivity. On the other hand, selectivity for ACRL-toxin was high irrespective of the bioassay techniques used.

I. Initial Site of Toxin Action

Mitochondria

Miller and Koeppe (1971) were the first to report the toxic effect of HMT-toxin on isolated mitochondria from susceptible T-cytoplasm-containing corn, whereas, mitochondria isolated from resistant N-cytoplasm-containing corn were not affected by the toxin. The toxin reduced the rate of malate oxidation, slightly increased the rate of succinate oxidation, and increased the rate of NADH oxidation. Later, it was suggested that stimulation of oxidation and inhibition of ATP synthesis indicate uncoupling of oxidative phosphorylation (Bednarski *et al.*, 1977; Payne *et al.*, 1980a), and that inhibition of malate oxidation, which is still coupled, may be a separate or secondary effect, perhaps by the toxin's interference with transport in or out of the matrix. Similar mitochondrial responses were observed when mitochondria isolated from young rough lemon leaves were treated with ACRL-toxin I (Kohmoto *et al.*, 1987). Effect of HMT-toxin on susceptible mitochondria was also confirmed by their inability to contract in the presence of 2-deoxyglucose (Malone *et al.*, 1978), and by the suppression of ATP levels in protoplasts observed three minutes after their treatment with the toxin (Walton *et al.*, 1979). Toxin-treated resistant mitochondria were able to contract in the presence of 2-deoxyglucose, and there was no suppression of ATP levels. Mitochondria from ACRL-toxin I-treated susceptible rough lemon leaves also failed to contract in the presence of 2-deoxyglucose, but the same was not true for mitochondria from toxin-treated resistant Dancy tangerine leaves (Kohguchi *et al.*, 1985 as referred by Kohmoto *et al.*, 1987). HMT-toxin also caused a decrease in Ca^{2+} accumulation in T-mitochondria, whereas a 100 times concentrated toxin caused little or no decrease in Ca^{2+} accumulation in N-mitochondria (Kimber and Sze, 1984). The decrease was due to increased permeability of the mitochondrial membrane to Ca^{2+}. The toxin seemed to increase the permeability of membranes to ions by forming a channel in the phospholipid bilayer (Holden *et al.*, 1985). It was suggested that the channel opening is caused by the action of the toxin on a membrane protein which is present in T-mitochondria but is absent or modified in N-mitochondria (Bervillé *et al.*, 1984; Bervillé and Paillard, 1984; Hartung *et*

al., 1987). Holden *et al.* (1985) suggested that in the presence of toxin, proton motive force was dissipated in T-mitochondria, whereas no change in proton motive force was observed in N-mitochondria. Dissipation of the force can explain the lack of oxidative phosphorylation, but as such cannot explain other mitochondrial responses. Bervillé *et al.* (1984) further reported that HMT-toxin induced release of NAD and coenzyme A from the mitochondrial matrix. In particular, they showed that in corn, normally NAD is transported into the mitochondrial matrix, and that the toxin inhibits this transport and in turn inhibits malate oxidation. Results of further studies suggested that ACRL- and HMT-toxins do not act as classical uncouplers, but seem to have two modes of action, one being inhibition of electron transport at Complex I and the second one being uncoupling of oxidative phosphorylation (Holden *et al.*, 1985; Holden and Sze, 1984; Kohmoto *et al.*, 1987). These observations also suggested that the toxins affect the electron transport chain of the inner membrane of mitochondria.

The conclusion, based on physiological responses, that mitochondria are the site of HMT- and ACRL-toxin action, was also supported by ultrastructural studies. Ultrastructural effects of HMT-toxin on susceptible tissue conducted using root tissue, isolated protoplasts, and mitochondria suggested that mitochondria were the first organelles to be affected (Aldrich *et al.*, 1977; Gregory *et al.*, 1980; York *et al.*, 1980). The ultrastructural affects on mitochondria included swelling, loss of matrix density, and unfolding of the inner membrane. No changes were observed in the mitochondria from resistant tissue. Similar ultrastructural changes were also observed in mitochondria from ACRL-toxin I- treated susceptible tissue (Kohmoto *et al.*, 1984) whereas mitochondria from the resistant tissue, and other organelles both from resistant and susceptible tissues, were not affected.

It has also been suggested that mitochondria are not the only site of HMT-toxin action (Arntzen *et al.*, 1973b; Daly and Barna, 1980; Gengenbach *et al.*, 1973). The plasma membrane was implicated as a target site of the toxin. The toxin caused a rapid host-specific inhibition of photosynthesis and transpiration. The inhibition was the result of closure of stomata, which in turn was associated with K^+ uptake by the guard cells

(Arntzen *et al.*, 1973a). The toxin also caused host-specific inhibition of ion uptake into apical root segments and leaf disks (Arntzen *et al.*, 1973a; Frick *et al.*, 1976, Mertz and Arntzen, 1977), depolarization of transmembrane electrical potential of root cells (Mertz and Arntzen, 1978), and leakage of electrolytes (Halloin *et al.*, 1973). These studies suggested that electrogenic ion pumps of the plasma membrane are also possible sites of toxin action. However, some of the studies described earlier were conducted using an impure toxin, and were not supported by ultrastructural studies. Furthermore, the physiological changes caused by the toxin can be explained by the rapid reduction in energy production by toxin-treated susceptible mitochondria. It is of interest to note that both ACRL-, and HMT- toxins contain similar polyol moieties in their molecules (Kohmoto *et al.*, 1987).

The receptor site of *A. alternata* tobacco pathotype toxin (AAT-toxin) has also been reported to be in the mitochondria (Kohmoto *et al.*, 1985; Nishimura, 1987). The toxin caused ultrastructural changes in the mitochondrion of a susceptible host, but did not affect its plasma membrane or chloroplasts (Kodama *et al.*, 1985, as referred by Kohmoto *et al.*, 1987). No other supporting evidence is yet available.

Plasma Membrane

Different physiological and ultrastructural studies suggest that the plasma membrane is the initial site of action of some toxins. AK-toxin caused a rapid loss of electrolytes (mainly K^+) from susceptible pear cells (Otani *et al.*, 1977; Nishimura and Kohmoto, 1983a); the leakage was detectable after a few minutes of toxin treatment and the leaf tissue lost 40% of the total K^+ within four h. Similar electrolyte losses were caused by ACTG-toxin (Kohmoto *et al.*, 1987), AF-toxin, (Maekawa *et al.*, 1984), and AM-toxin (Kohmoto *et al.*, 1976; Nishimura and Kohmoto, 1983b; Nishimura *et al.*, 1976). The observations suggested that the toxins disturb the integrity of the plasma membrane of susceptible cells and that the plasma membrane is the initial site of toxin action (Kohmoto *et al.*, 1987; Maekawa *et al.*, 1984).

Another approach to determine the initial site and mode of action of toxin has been the use of metabolic inhibitors. Metabolic inhibitors protected susceptible pear tissue against AK-toxin action (Otani *et al.*, 1985a). Pre-treatment of susceptible pear tissue with sulfhydral reagents inhibited electrolyte leakage as well as the development of necrosis, whereas cycloheximide and copper chelating agents inhibited necrosis only. This suggested that the toxin has two different phases of action, plasma membrane damage associated with sulfhydral-containing molecules, followed by cell death associated with *de novo* synthesis of proteins and copper containing molecules.

Electrophysical experiments can also be used to demonstrate that the plasma membrane is the initial site of toxin action. AF- and AK- toxins caused an instantaneous reduction in membrane potential of susceptible strawberry and pear cells, respectively (Kohmoto *et al.*, 1987; Namiki *et al.*, 1986a). The depolarization was due to the loss of the active component of membrane potential, suggesting that the electrogenic H^+-pump might be selectively damaged by the toxin. Resistant cells showed no such depolarization of membranes. Depolarization of membrane potential was also observed when susceptible sugarcane tissues were treated with HS-toxin (Schröter *et al.*, 1985). However, it was suggested that the toxin acts on the plasma membrane at a site other than the H^+-pump, because in the presence of the toxin the pump can be activated by light and fusiccocin.

Time-course study of ultrastructural changes observed in toxin-treated susceptible tissue is another widely used criterion for determining initial site of toxin action. Park (1977) reported that AK-toxin caused invaginations in the plasma membrane of susceptible pear tissue one h after toxin treatment. Lomasome-like vesicles and membrane fragments observed between the cell wall and the invaginated plasma membrane were thought to have originated from the plasma membrane, whereas similarly placed amorphous material was thought to have originated from the cell wall. Results of this and other ultrastructural studies suggested that the plasma membrane is the first site to be affected by the toxin (Park, 1976, 1977; Park *et al.*, 1976).

Ultrastructural effects of ACTG-toxins A and B (Kohguchi *et al.*, 1984; Kondoh *et al.*, 1984, both as referred by Kohmoto *et al.*, 1987), AF-toxins I and II, AK-toxin,

AAL-toxin, and AM-toxin I (Park *et al.*, 1981a), were similar to that of AK-toxin (Park, 1977). Although the ultrastructural effects were similar, the conclusions drawn were different from those in the earlier report. It was concluded that the interface between the cell wall and plasma membrane is the site of initial effect of AK-toxin (Kohmoto *et al.*, 1987; Park *et al.*, 1981a). At the same time, it was reported in another study that there are differences in the effects of AK-toxin on susceptible intact petals and its protoplasts (Otani *et al.*, 1981). The toxin caused modifications of the plasma membrane of cells of intact tissue, but did not effect the plasma membrane of isolated protoplasts from the same host. These results suggested that the cell wall and plasmodesmata are necessary, at least, for one of the sites of toxin action (Park *et al.*, 1981a), and plasma membrane invaginations may be the effect of degradation of cell wall and plasmodesmata. Otani *et al.* (1985b) also reported invagination of the plasma membrane and degradation of the cell wall.

Since ultrastructural effects of ACTG-toxin A, AF-toxins I and II, and AM-toxin I were similar to those of AK-toxin (Park *et al.*, 1981a, 1981b), it appears that the sites of initial effects of these toxins are also at the interface between the cell wall and the plasma membrane (Kohmoto *et al.*, 1987; Nishimura and Kohmoto, 1983a; Park *et al.*, 1977, 1981a, 1981b).

Earlier ultrastructural studies indicated that the first effect of HV-toxin was on the cell wall of the susceptible oat tissue (Hanchey *et al.*, 1968; Luke *et al.*, 1966). Wheeler (1975, 1976) also supported the hypothesis that the initial site of HV-toxin action is the cell wall. He based his argument on a study of oat coleoptile growth (Saftner and Evans, 1974). It was agreed that a decline in the rate of electrolyte leakage after 5 min of toxin treatment would not be observed if the initial effect of the toxin is disruption of protoplast permeability (Wheeler, 1976). He further indicated that the growth pattern of susceptible coleoptile, pattern of electrolyte leakage, and pattern of changes in electrochemical potential also suggest that the initial effect of the toxin was on some cell wall component. However, the effect shown by a high concentration of the impure toxin may also be due to an impurity (Yoder, 1980). Furthermore, HV-toxin action site on the cell wall, if present, must be independent from another site in the cell since protoplasts are known to be killed

by the toxin (Briggs *et al.*, 1984; Rancillac *et al.*, 1976; Samaddar and Scheffer, 1968). Results of later studies supported the hypothesis that the receptor site for HV-toxin is in the plasma membrane of the susceptible host. Pre-treatment of susceptible oat tissue with metabolic inhibitors such as cyclohexamide and sulfhydral-binding reagents protected the tissue from the effects of the toxin, but the effect was reversed 48 h after removal of the compound (Gardner and Scheffer, 1973). The results suggested that HV-toxin effects a sulfhydral-containing membrane-bound protein that is constantly turned over (Daly, 1981). However, Daly (1981) cautioned that reversal effect of the toxin observed by Gardner and Scheffer (1973) is inconsistent with the lethal effects of the lower toxin concentrations obtained by Samaddar and Scheffer (1968).

The main evidence for initial action site of HV-toxin in the plasma membrane is based on the speed by which the characteristics of the plasma membrane in toxin-treated susceptible tissue are changed (Yoder, 1980). The toxin caused rapid depolarization of membrane potential of the susceptible cells, whereas the resistant cells were not affected (Gardner *et al.*, 1974; Novacky and Hanchey, 1974). The presence of HV-toxin receptor sites on the plasma membrane was indicated by studies conducted using isolated cells and protoplasts. Generally, rapid killing of susceptible protoplasts was observed when they were treated with HV-toxin (Briggs *et al.*, 1984; Rancillac *et al.*, 1976; Samaddar and Scheffer, 1968), but in one study, 3-4 h were required for protoplast mortality (Hawes, 1983a). Cells isolated with or without cell walls showed similar death rates when treated with HV-toxin (Hawes, 1983a), and leaf protoplasts showed toxin sensitivity similar to that of leaf tissue and intact roots (Briggs *et al.*, 1984). These observations suggested that cell wall is not necessary for HV-toxin action.

Other Sites

Physiological and ultrastructural studies suggested that there is also a second primary site of AM-toxin action. Chlorophyll-lacking tissue such as filaments, flesh cells, petals, and styles from susceptible apples were not affected by AM-toxin I, whereas a much lower toxin concentration damaged green tissue (Maeno *et al.*, 1980, as referred by Kohmoto *et*

al., 1987). The toxin also caused a reduction in the chlorophyll content of susceptible, but not of resistant, leaves. AM-toxin I inhibited light CO_2 fixation in the susceptible leaf tissue, but for similar inhibition in the resistant leaf tissue, a 1000 times higher toxin concentration was required (Kohmoto *et al.*, 1982a; Nishimura and Kohmoto, 1983a). Results of physiological changes were supported by ultrastructural studies. The first effect of AM-toxin I in the susceptible apple leaves was the appearance of disrupted grana-derived vesicles and membranes in the chloroplast matrix along with invaginated plasma membrane (Nishimura *et al.*, 1982; Park *et al.*, 1981a, 1981b). The results of these and other studies taken together suggested that AM-toxin has two primary sites of action, one is the chloroplast and the other is the cell membrane/cell wall region of susceptible cells.

In contrast to other host-specific *Alternaria* toxins, *A. alternata* tomato pathotype produced AAL-toxins that did not cause rapid electrolyte leakage from susceptible tissue (Kohmoto *et al.*, 1982b; McFarland and Gilchrist, 1982). Electrolyte leakage was not observed until necrosis began to appear, usually 30-40 h after toxin treatment. Ultrastructural studies indicated that the first effect of the toxin was on the mitochondria and the endoplasmic reticulum of susceptible leaf cells (Park *et al.*, 1981a). Mitochondria were swollen, their matrix was leached, and there was a reduction in the number of cristae. The toxin also caused the endoplasmic reticulum to swell and vesiculate, but it did not induce any modifications of plasma membrane. Although ultrastructural changes in mitochondria were similar to those caused by HMT-toxin on mitochondria of T-cytoplasm corn (Aldrich *et al.*, 1977; Gregory *et al.*, 1980), there was no effect on respiration of mitochondria isolated from susceptible tomato tissue (Kohmoto *et al.*, 1987). Presence of an L-aspartate moiety in the structure of AAL-toxin indicated that site of toxin action may be linked with the uptake or metabolism of aspartate, and the results of further studies indicated that the toxin causes an inhibition of aspartate transcarbamoylase, possibly by acting as an antimetabolite (McFarland and Gilchrist, 1981; Gilchrist, 1983).

Proposed Sites

The mode of action of HS-toxin is controversial. Strobel and his colleagues published a series of papers suggesting that the toxin binds to a plasma membrane protein of susceptible sugarcane cells. No such binding of toxin to resistant sugarcane cells was observed (Strobel, 1973, 1974, 1975, Kenfield and Strobel, 1981a, 1981b). Consequently, there has been a widespread criticism of this work. Daly (1982) suggested that lines of evidence provided in these studies have major experimental deficiencies. Livingston and Scheffer (1984b) reported that toxin analogues protected sensitive sugarcane tissue against active toxin. They suggested that the analogues interact with a toxin receptor, thereby reducing toxin interaction with the receptor. Furthermore, the interaction between the toxin and the receptor is qualitatively different from that between the analogues and the receptor, and only toxin-receptor interaction leads to electrolyte leakage. The toxin also caused a rapid and substantial inhibition of dark CO_2 fixation in the susceptible sugarcane cells (Duvick *et al.*, 1984), and the toxin analogues protected the tissue from the inhibitory effects of the toxin (Duvick *et al.*, 1984, Livingston and Scheffer, 1984a, 1984b). A very low HS- toxin concentration (50μM) also caused depolarization of membrane potential of susceptible sugarcane. The depolarization started after a lag period of 4-10 min, depending upon toxin concentration (Schröter *et al.*, 1985). They postulated that the toxin causes a loss of H^+ gradient across the plasma membrane and suggested that it is unlikely that the H^+ pump is the site of toxin action, because in the presence of the toxin, the pump can be activated by light and fusicoccin. Duvick *et al.* (1984), referring to the results of Schröter *et al.* (1985), suggested that the inhibitory effect of the toxin on dark CO_2 fixation by susceptible sugarcane is due to perturbation of H^+ gradient across the plasma membrane.

Effects of HC-toxin on susceptible corn tissue are also quite different from those of other host-specific toxins. The toxin caused a rapid but transient increase in electronegative potential, whereas HV and PC-toxins caused decreases in the electronegative potential (Gardner *et al.*, 1974). Futhermore, unlike many other host-specific toxins, no loss of electrolytes from susceptible tissue was detected within 5 to 6 h after toxin treatment, but eventually the cells became necrotic and leaky (Scheffer and Livingston, 1984; Yoder and

Scheffer, 1973a). The toxin-treated susceptible corn roots took up and retained more nitrates (Yoder and Scheffer, 1973b). The increased nitrate accumulation was not due to decreased nitrate metabolism, or decreased nitrate leakage, but due to increased nitrate uptake. It is not clear whether these effects are due to the effect of the toxin on the plasma membrane or on energy-yielding processes (Daly, 1981; Scheffer and Livingston, 1984; Yoder, 1980), and as yet there is no proposed mechanism of HC-toxin action.

J. Use of Fungal Toxins as Herbicides

Many different methods are used to control weeds infesting field crops, orchards and pastures. Application of chemical herbicides has been one of the preferred methods, and herbicides have become even more popular due to the recent emphasis on reduced or no-till agriculture (Duke, 1986a). As a result, many highly active and selective herbicides have been developed (Fischer and Bellus, 1983). However, researchers are now emphasizing biological control of weeds (Anonymous, 1986a; Charudattan and Walker, 1982; Delfosse, 1985; Fischer and Bellus, 1983). There are many reasons why biological control of weeds is becoming popular. Agrologists are now realizing not only the importance of an integrated approach for management of weeds (Shaw, 1982), but also the higher costs of mechanical, hand labour, and in some cases chemical weed control (Charudattan, 1986). Furthermore, public awareness about problems, such as herbicide residue in soils and crops, possible air and water pollution, and human exposure during herbicide application, associated with synthetic chemical herbicides (McWhorter and Chandler, 1982), also encouraged researchers to use natural enemies of weeds as biocontrol agents. Both plant pathogens and insects have been used as biocontrol agents of weeds (Delfosse, 1985; Freeman *et al.*, 1974; Harris, 1980; Julien, 1982). Although different classes of plant pathogens including fungi, bacteria, viruses and nematodes have been used with varying degrees of success throughout the world (Templeton, 1982), fungi have been the most common biocontrol agents (Freeman, 1980). *Puccinia chondrillina* Bubàk and Syd. has been very successful in controling *Chondrilla juncea* L. (skeltonweed) in Australia (Cullen *et al.*, 1973; Hasan, 1974). *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene* has been very effective in controlling

Aeschynomene virginica (L.) B.S.P. (Joint vetch) in rice and soybean (Templeton *et al.*, 1978), and spores of this fungus are commercialized as the mycoherbicide called Collego™ by Upjohn Co. *Phytophthora palmivora* Butler is another successful mycoherbicide marketed as DeVine™ by Abbott Laboratories for use against *Morrenia odorata* Lindl. (stranglevine) in citrus orchards (Kenney, 1986; Ridings, 1986; Ridings *et al.*, 1978). Although there are some success stories of biological control of weeds by organisms, generally they fail to adequately control weed populations because all fungi used as mycoherbicides are slow to spread, have low virulence, and require narrow environmental conditions (Templeton *et al.*, 1979). Furthermore, the most important concern is that because of genetic variation in the organisms, progeny of the organism may become parasites of non-target plants (Freeman and Charudattan, 1985; Templeton *et al.*, 1979). Risks and efficiency problems with bioherbicides, and environmental concerns about synthetic chemicals can be considerably reduced by using natural phytotoxic metabolites, or their synthetic analogues, produced by the microorganisms (Fischer and Bellus, 1983; Freeman and Charudattan, 1985; Misato and Yamaguchi, 1984). Gene cloning technology may help to increase the virulence of the pathogen by cloning and transferring genes responsible for toxin production to a non-toxin producing pathogen specific for the target weed (Turgeon and Yoder, 1985), but there are many big "ifs" involved, and relaxations in the U.S. Environmental protection Agency guidelines for registration of bioherbicides are not available to genetically engineered organisms (Freeman and Charudattan, 1985). In two recent articles, Duke (1986a, 1986b) has discussed many important points regarding the usage of biologically produced phytotoxins as herbicides. He pointed out that the phytotoxins are better weed control agents than living organisms because "a) they generally have a longer shelf life and require much less storage space, b) the logistics of formulation and application are usually simpler, c) there is no danger of disease spread to nontarget species, d) results are not as dependent on environmental conditions, e) the efficacy of particular application rates can be more accurately predicted, and f) they may often be less expensive to produce." He added further that there is a need a) for new chemical classes of herbicides because weeds are becoming resistant to some important herbicides such as

s-triazines and dinitroanilines, and b) for more selective herbicides because some crops are infested by closely related weeds, such as wild oats in oats, red rice in rice, and shattercane in sorghum. However, specificity of an herbicide may also work against its commercialization unless the herbicide is very effective against an important weed. Plant pathogens not only provide chemicals with different chemistries than those of the traditional herbicides (Cutler, 1986; Duke, 1986a; Fischer and Bellus, 1983), but also provide specific, efficaceous and environmentally safe herbicides because they coevolved and have close association with host plant species (Duke, 1986b). The cost of discovering a herbicide is also reduced because there are fewer plant pathogen-produced chemicals to screen than in the traditional approach (Duke, 1986a). Naturally occurring compounds also enjoy relaxed registration guidelines (Freeman and Charudattan, 1985; Mortensen, 1986).

Microorganisms are known to produce both host-specific and nonhost-specific toxins. Initial interest in pathogen-produced compounds with phytotoxic actions was to explain the biochemical basis of the pathogenicity of plant pathogens of economically important crops (Scheffer and Livingston, 1984). Systematic and emperical evaluation of compounds produced by microorganisms revealed a number of non-specific toxins with herbicidal activity (Fischer and Bellus, 1983). Some of these compounds such as tentoxin and zinniol are selective enough and can be used as herbicides because they are not toxic to some commercially important crops (Duke, 1986a). Zettler and Freeman (1972) suggested the use of *A. eichorniae* Nag Raj and Ponnappa toxin to control *Eichornia crassipes* (Mart.) Solms (water hyacinth), a pernicious aquatic weed. Bostrycin and 4-deoxybostrycin isolated from the culture filtrates of the fungus were found to be non-specific toxins with potential as broad-spectrum herbicides (Charudattan and Rao, 1982; Robeson *et al.*, 1984). Zinniol, a nonhost-specific toxin produced by many *Alternaria* species (Cotty and Misaghi, 1984) shows phytotoxic activity against many plants from different families (White and Starratt, 1967), but is also known to enhance chlorophyll retention in the leaves of barley, wheat, and oats (Robeson and Strobel, 1984). Tentoxin produced by *A. alternata* is effective against a large number of weeds including johnson grass and does not cause chlorosis in many commercially important crops, including soybean

and maize (Duke, 1986b; Durbin and Uchytil, 1977). Bialophos, a nonhost-specific toxin produced by *Streptomyces viridochromogenes* (Krainsky) Waksman and Henrici was the first herbicide to be produced by fermentation, is registered in Japan (Tachibana *et al.*, 1986), and is expected to be used against wide range of weeds occurring between rows of crops in orchards.

Host-specific toxins are key factors in the disease development because they are biochemical bases for pathogenicity, host specificity of microorganisms and disease resistance (Scheffer and Livingston, 1984; Scheffer and Samaddar, 1970). All reported host-selective toxins, except one, were discovered because they were produced by pathogens of commercially important crops. Realization of the importance of host-selective toxins in disease development has focussed attention on isolation and characterization of toxins from weed pathogens (Duke, 1986a). There are a few recent reports of preliminary investigations of toxins isolated from weed pathogens. While some of these toxins may prove to be potentially effective herbicides against some important weeds, at present, there is only one report of a selective toxin from a weed pathogen. The toxin, Bipolaroxin, is produced by *Bipolaris cynodontis* (Marig.) Shoem, a fungus infecting a select range of weeds, including bermuda grass (Anonymous, 1986b). The toxin has the potential of being used as a herbicide against bermuda grass. Toxins from two other weed pathogenic fungi *Bipolaris sorghicola* (Lefebvre and Sherwin) comb. nov. (Peña-Rodriguez *et al.*, 1987), and a *Xylaria* sp. Hill ex Greville (Karl *et al.*, 1986) show herbicidal activity against johnson grass and *Euphorbia heterophylla* L., respectively. However their possible effects on other plant species are currently unknown. Search for phytotoxins from fungal pathogens of weeds may yield many more host-specific and nonhost-specific toxins, and some of these toxins may prove to be good herbicides.

K. Objectives of the Thesis

The objectives of this thesis were (1) to develop a new method for purifying the toxin produced in culture by *A. brassicae* and study some of its chemical characteristics, (2) to study some of the biological characteristics of the toxin to evaluate its role in disease

development, (3) to develop a sensitive, fast, and simple bioassay method for the toxin, (4) to study the initial site of toxin action, and (5) to evaluate its efficacy as a herbicide to control some important cruciferous weeds in cereal crops.

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II. Purification, chemical characterization and host-specificity of the toxin produced by *Alternaria brassicae*

A. Introduction

The black spot caused by *Alternaria brassicae*[1] (Berk.) Sacc. is a major disease of rapeseed (*Brassica campestris* L. and *Brassica napus* L.) in Western Canada (Petrie, 1975) and around the world (Weiss, 1983). The pathogen infects all aerial plant parts, reducing the photosynthetic area and accelerating senescence and defoliation. Fruit infection results in premature shattering, leading to seed losses during harvesting. Precise yield loss estimates have not been made, but in artificially *A. brassica*-inoculated field crops, yield losses of 63% and 42% have been reported for *B. campestris* cv. Span and *B. napus* cv. Zephyr, respectively (Degenhardt *et al.*, 1974). The oil and protein contents of the seed are also significantly reduced, particularly in *B. campestris*. Despite the importance of the disease, little information on the mode of pathogenesis is available.

Several species of *Alternaria* Nees ex Fr. are reported to produce toxins. There are at least six host-parasite combinations, involving species of *Alternaria*, where the toxins produced are host-specific (Nishimura and Kohmoto, 1983; Yoder, 1980). Host-specific toxins are toxic only to hosts of the pathogen that produce them and can reproduce the disease symptoms in the absence of the pathogen (Scheffer and Livingston, 1984). Some *Alternaria* species produce host non-specific toxins such as alternariols, alternaric acid and zinnoil (Cotty and Misaghi, 1984; Stoessl, 1981).

In an earlier study it was reported that crude culture filtrates of *A. brassicae* are toxic to *B. campestris* var. *yellow sarson* seedlings (Husain and Thakur, 1966). Later, Degenhardt (1978) reported that the fungus produced two groups of non-specific toxins *in vitro* which are capable of causing disease symptoms in leaves. However, the final preparations used for identification of the toxins were described as semipurified

[1]This chapter has been published as follows: Bains, P.S., and J.P. Tewari, 1987. Purification, chemical characterization and host-specificity of the toxin produced by *Alternaria brassicae*. Physiological and Molecular Plant Pathology 30, 259-271.

(Degenhardt, 1978). In the present paper, a chromatographic procedure for purification of the toxin produced by *A. brassicae* is described. This paper also describes some of the chemical characteristics of the purified toxin, and provides evidence which indicates that the activity of this toxin is host-specific. A brief report on certain preliminary observations made during this study was published earlier (Bains and Tewari, 1985). Chemical characteristics of the purified toxin suggest that the toxin is identical to the one isolated primarily by partitioning with organic solvents from culture filtrates of *A. brassicae*, in a concurrent study on metabolites produced by the fungus (Peña-Rodriguez, 1985). In that study (Peña-Rodriguez, 1985) the toxin was identified as destruxin B which has been reported to be produced by *Metarhizium anisopliae* (Metschn.) Sorokin, a fungus pathogenic on insects (Pais *et al.*, 1981; Tamura *et al.*, 1964).

B. Materials and Methods

Bioassay of toxic fractions during the isolation procedure

Detached[2] third or fourth leaves from plants of *B. napus* cv. Altex [growth stages 3.3 to 4.2 (Harper and Berkenkamp, 1975)] grown in the greenhouse were used for toxin bioassay. An area of approximately 5 mm^2 on the upper leaf surface was gently scratched with the tip of a Pasteur pipette, and 20 μl of the test solution were applied to it. The leaves were then incubated on a moist filter paper in a Petri dish, which was kept on a laboratory bench under continuous fluorescent light. Chlorosis and necrosis due to the toxic fraction appeared within 48 h of incubation. No such symptoms in the leaf appeared when the control test fraction was applied. Toxic activity was bioassayed after each purification step.

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[2]Thanks are due to Drs W.A. Ayer and L.M. Peña-Rodriguez for supplying a sample of Destruxin B and helpful discussions and to Dr S. Zalik and Mr B. Zytaruk for facilitating HPLC analyses.

Fungal culture for toxin production

Alternaria brassicae was isolated from naturally infected rapeseed leaves collected from Barrhead, Alberta and was used throughout in this study. Leaves were surface-sterilized, incubated in a moist chamber for four days and single spore isolations were made on V8 juice agar medium containing 0.004% Rose Bengal, where the pathogen sporulated profusely (Degenhardt *et al.*, 1974). The fungus for toxin production was grown in Roux bottles (50-100/ batch) containing 100 ml of liquid V8 juice medium [250 ml of V8 juice, 1000 ml of double distilled water and 0.936 g of $CaCO_3$ (Degenhardt, 1978)]. The medium in each bottle was inoculated with 1 ml of spore suspension (5 x 10^4 spores/ml) obtained from 10- to 14-day-old cultures. The bottles were kept at 20°C with a daily light period of 8 h at approximately 60 lux. Sterile uninoculated V8 juice medium was used as control.

Purification of toxin

Twenty-seven-day-old fungal cultures were filtered through 4 layers of cheesecloth. The filtrate (5-10 l) obtained was successively passed through filter paper No. 202 Reeve-Angel, No. 3 Whatman, and No. 1 Whatman. Further clarification was obtained by passing through 0.8 μm and 0.22 μm Sterivex-GS filter cartridges (Millipore Corp.) with the help of a peristaltic pump. All filtrations were carried out at 4°C. The filtrate was concentrated under reduced pressure to 10% of the original volume (all concentrations were carried out at 45°C), and the concentrated filtrate was filtered to remove the precipitates which appeared upon concentration. Fifty millilitres of this preparation were applied to a large (4 x 180 cm) column of Sephadex G-50 coarse (Sigma Chemical Co.), which was previously equilibrated with water. Flow rates of this as well as other columns used were adjusted to 1 ml/min. The column was washed with water and 20-ml fractions were collected. The toxic fractions were pooled, concentrated to 20 ml, and placed on a smaller (4.5 x 42 cm) column of Sephadex G-25 medium (Sigma Chemical Co.), which was previously equilibrated with water. The column was developed with water and 10 ml

fractions were collected. Toxin-containing fractions were pooled, and the toxin was adsorbed on activated charcoal (Bio-Rad Labs.) by shaking the pooled fraction with charcoal (6 g/l of the original filtrate volume) on a wrist action shaker overnight at 4°C. The charcoal suspension was centrifuged at 18 800 g for 20 min, the supernatant discarded and the charcoal pellet resuspended in 50 ml of water. The suspension was recentrifuged and the supernatant discarded. The toxin was desorbed from charcoal by shaking it with 20 ml of ethyl acetate for 1 h at room termperature. Charcoal was allowed to settle, and the ethyl acetate fraction was pipetted out. Desorption with ethyl acetate was repeated 4 times. All ethyl acetate fractions were pooled and centrifuged at 18 800 g for 30 min. Charcoal after ethyl acetate desorption was similarly washed 3 times with water-saturated *n*-butanol and the washings were pooled and centrifuged at 18 800 g for 30 min.

The pooled ethyl acetate fraction was flash evaporated to dryness, resuspended in 1.5 ml of benzene: acetone (5:1), and loaded onto a 1.5 x 42 cm column of silica gel (40-140 mesh) (J.T. Baker Chemical Co.) which was previously equilibrated with benzene:acetone (5:1). The silicic acid column was developed with 200 ml of benzene:acetone (5:1), followed by 200 ml of benzene:acetone (4:2). Five-millilitre fractions were collected, flash evaporated to dryness, and suspended in 1 ml of water for bioassaying. All toxic fractions were pooled together, and the resultant fraction was used to study its toxic properties.

The butanol fraction was flash evaporated to dryness, resuspended in 1.5 ml of water, and applied to a Sep-Pak C_{18} cartridge (Waters Associates). The Sep-Pak C_{18} cartridge was successively washed with 10 ml of 0, 20, 40, 50, 55, 60, and 70% methanol in water. Ten-millilitre fractions were collected, flash evaporated to dryness, and resuspended in 1 ml of water for bioassay. The Sep-Pak C_{18} cartridge was used because, in preliminary experiments, silicic acid column chromatography of the butanol fraction resulted in poor toxin recovery, most probably due to its spreading in the column.

The pooled toxic fraction from the silicic acid column and toxic fraction from the Sep-Pak C_{18} cartridge were further purified on a Varian 5000 high pressure liquid

chromatograph equipped with a Serva C_{18} reverse phase column (7.1 x 500 mm). The solvent system was similar to that used by Walton and Earle (1984a). Solvents A and B consisted of 0.05% trifluoroacetic acid in water, and 0.05% trifluoroacetic acid in acetonitrile (HPLC grade, Caledon Labs), respectively. A linear gradient of 0-60% of B was achieved in 30 min, at a flow rate of 4 ml/min. Eluent from the column was monitored at 235 nm with a Varian variable wavelength detector UV-50. The fraction in the peak was collected, flash evaporated to dryness, resuspended in water, and bioassayed. Toxin purified by HPLC was used to study its chemical characteristics. HPLC of destruxin B (obtained from Drs W.A. Ayer and L.M. Peña-Rodriguez) and of a 1:1 mixture of destruxin B and the toxin isolated in this study were performed similarly.

Chemical characteristics of the toxin

The reaction of toxin and hydrolysed toxin with ninhydrin was studied according to the procedure of Spies (1957). The toxin was hydrolysed with 6 N HCl at 110°C for 40 h in a glass tube with a teflon-lined screw cap. Amino acids were separated and identified on a Beckman amino acid analyser model 121 MB. Reaction of destruxin B with ninhydrin was also studied similarly.

The fast atomic bombardment (FAB) mass spectrum of the toxin was recorded on a KRATOS MS-9 spectrometer on a stainless steel probe. Data were recorded as relative intensity (m/z). High resolution mass spectrum (HRMS) was recorded on a KRATOS MS-50 spectrometer equipped with data systems DS-55.

Proton nuclear magnetic resonance (^{1}H NMR) spectrum of the toxin in $CDCl_3$ was measured on a Bruker WH-400 spectrometer equipped with computer system ASPECT 2000. Tetramethylsilane was used as an internal standard.

Inoculations and treatments of brassicas with *Alternaria brassicae* and the toxin

Detached third or fourth leaves from 4-week-old plants of *B. campestris* var. *yellow sarson*, var. *toria*, and cv. Candle, *B. napus* cv. Altex, *B. juncea* (L.) Cosson cv.

Lethbridge 22A, *B. nigra* (L.) Koch, *B. hirta* Moench cv. Gisilba, and *B. campestris* ssp. *rapifera* were inoculated with *A. brassicae* spore suspension and treated with the toxin. An area of approximately 3 mm^2 on the upper leaf surface was gently scratched with the tip of a pasteur pipette, and a 50μl drop of spore suspension (5 x 10^4 spores/ml), or 20 μl of toxin solution were placed on the scratched area. Four leaves from each plant type were used for spore inoculations, and each leaf was inoculated at 4 points, two on either side of the mid-rib. The experiment was repeated twice. The toxin obtained from silicic acid column was diluted to 240, 120, 60, 30 and 15 μg/ml with water. Four half-leaves of each plant type were treated with each dilution of the toxin. The toxin was applied at 2 points on each half-leaf. The corresponding undiluted fraction from liquid V8 juice medium was used as control. The experiment was repeated twice. Leaves were placed on a moist filter paper in a Petri dish, which was kept on a laboratory bench under continuous fluorescent light. Reactions of the leaves were noted after 72 h of incubation.

Treatments of nonhosts of *Alternaria brassicae* with the toxin

To test the response of nonhosts of *A. brassicae* to the toxin, nine plant genera belonging to five families were fed the toxin through their vascular systems. Detached leaves or young stems bearing leaves from 4-week-old plants of *Avena sativa* L. (oats), *Cucumis sativus* L. (cucumber), *Hordeum vulgare* L. (barley), *Linum usitatissimum* L. (flax), *Lycopersicon esculentum* Mill. (tomato), *Secale cereale* L. (rye), *Triticum aestivum* L. (wheat), *Vigna unguiculata* (L.) Walp. (cowpea), and *Zea mays* L. (corn) were treated with the toxin using the following method. A 20 μl droplet of 300 μg/ml toxin was placed on Parafilm and the freshly cut petiole, stem or leaf lamina (in the case of barley and wheat) was dipped in it. The toxin was taken up into the plant part within a few min. The treated materials were incubated in a plastic bag with their cut ends immersed in water contained in a test tube. *Brassica campestris* var. *yellow sarson*, a host of the fungus, was similarly treated with 300 and 30 μg/ml of the toxin. The effects on treated plant species were recorded after 72 h of incubation under continuous fluorescent light at room

temperature. This method of toxin treatment was necessitated because, in preliminary experiments, scratching of the detached leaves of some non-hosts of *A. brassicae* resulted in necrosis and chlorosis upon incubation in a moist chamber, confounding the results of toxin testing.

Treatments of *Brassica nigra* and *Brassica campestris* ssp *rapifera* with destruxin B and the toxin

Detached leaves from 5-week-old plants of *B. nigra* and *B. campestris* ssp *rapifera* were treated with 120, 30 and 7.5 μg/ml of destruxin B and the toxin and with distilled water. Similar concentrations of destruxin B and the toxin were applied on opposite half-leaves. Four half-leaves were used for each concentration. The methods of treatment of leaves with test solutions and their incubation thereafter were similar to those used for the treatment of *Brassica* species with the toxin. This experiment was repeated once.

C. Results

Purification of the toxin

The crude concentrated culture filtrates of *A. brassicae* produced symptoms on *B. napus* cv. Altex leaves similar to those produced by the pathogen. Chromatography of these filtrates on a Sephadex G-50 coarse column resulted in a single toxic peak. Further chromatography of this toxic fraction on a Sephadex G-25 column resulted in elution of a toxic peak after the void volume of the column. The toxic fraction desorbed from charcoal with ethyl acetate eluted as a single toxic peak from the silicic acid column. Small amounts of yellow impurities desorbed from charcoal were retained in the top 1 cm of the column. Chromatography of the butanol fraction on Sep-Pak C_{18} cartridge resulted in the elution of a toxic fraction with 55% methanol in water. The yellow colored impurities desorbed from charcoal with butanol were eluted with lower methanol concentration, and the toxic fraction obtained was colorless.

HPLC of toxin containing fractions both from the silicic acid column and Sep-Pak C_{18} cartridge resulted in a single symmetrical peak at 26 min [Figs. II-1(a) and II-1(c)]. Corresponding control fractions did not show any peak at that retention time [Figs. II-1(b) and II-1(d)]. With a modified solvent system [addition of 1.5% (v/v) isopropanol to both solvents A and B], the toxin fraction again ran as a single symmetrical peak with a slightly different retention time. The fraction appearing as a peak was collected and its bioassay showed it to be toxic. Both destruxin B and a 1:1 mixture of destruxin B and the toxin isolated in this study resulted in a single symmetrical peak at 26 min.

Chemical characterization of the toxin

The ninhydrin test applied to the HPLC-purified toxin from both ethyl acetate and butanol fractions was negative. However, after hydrolysis of the toxin, a positive ninhydrin test was observed. Destruxin B also showed similar reactions with ninhydrin. Amino acid analysis of the hydrolysed toxin yielded isoleucine, proline, and β-alanine in the ratio of 1:1:1.

The FAB mass spectrum of HPLC-purified toxins from both ethyl acetate and butanol fractions showed protonated molecular ion (MH^+) peak at m/z 594. The molecular weight obtained from the FAB spectrum was confirmed by HRMS, which gave an exact mass of 593.3791 with a molecular formula $C_{30}H_{51}N_5O_7$ (calculated molecular weight = 593.3788).

Proton nuclear magnetic resonance spectrum indicated the presence of two amide, two *N*-methyl, seven methyl, and five α-carbon protons (Fig. II-2). The coupling pattern matches the expected coupling pattern of the structure of destruxin B reported earlier (Peña-Rodriguez, 1985; Tamura *et al.*, 1964), and the spectrum of the toxin is identical to that of Destruxin B (Peña-Rodriguez, 1985).

Fig. II-1. Representative HPLC elution profiles of toxic and control fractions eluted from silicic acid column and Sep-Pak C_{1}, cartridge. The solvent system used consisted of 0.05% trifluoroacetic acid in water (A), and 0.05% trifluoroacetic acid in acetonitrile (B). A linear gradient of 0-60% of B was obtained in 30 min at 4 ml/min (a) Active fraction, and (b) control fraction from the silicic acid column. (c) Active fraction, and (d) control fraction from Sep-Pak C_{1}, cartridge.

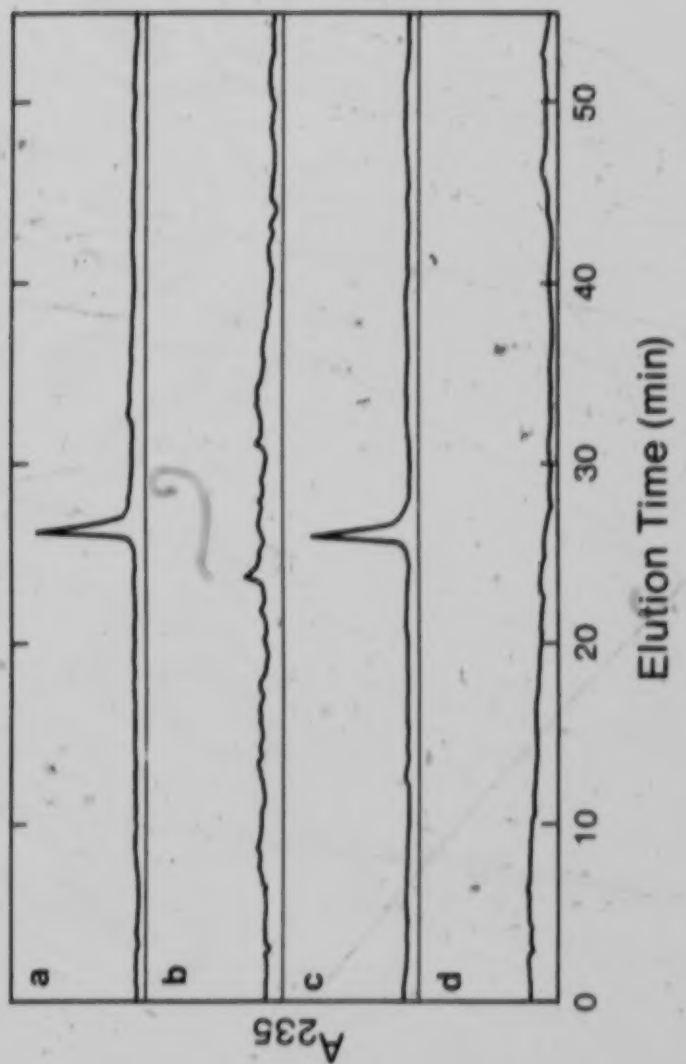
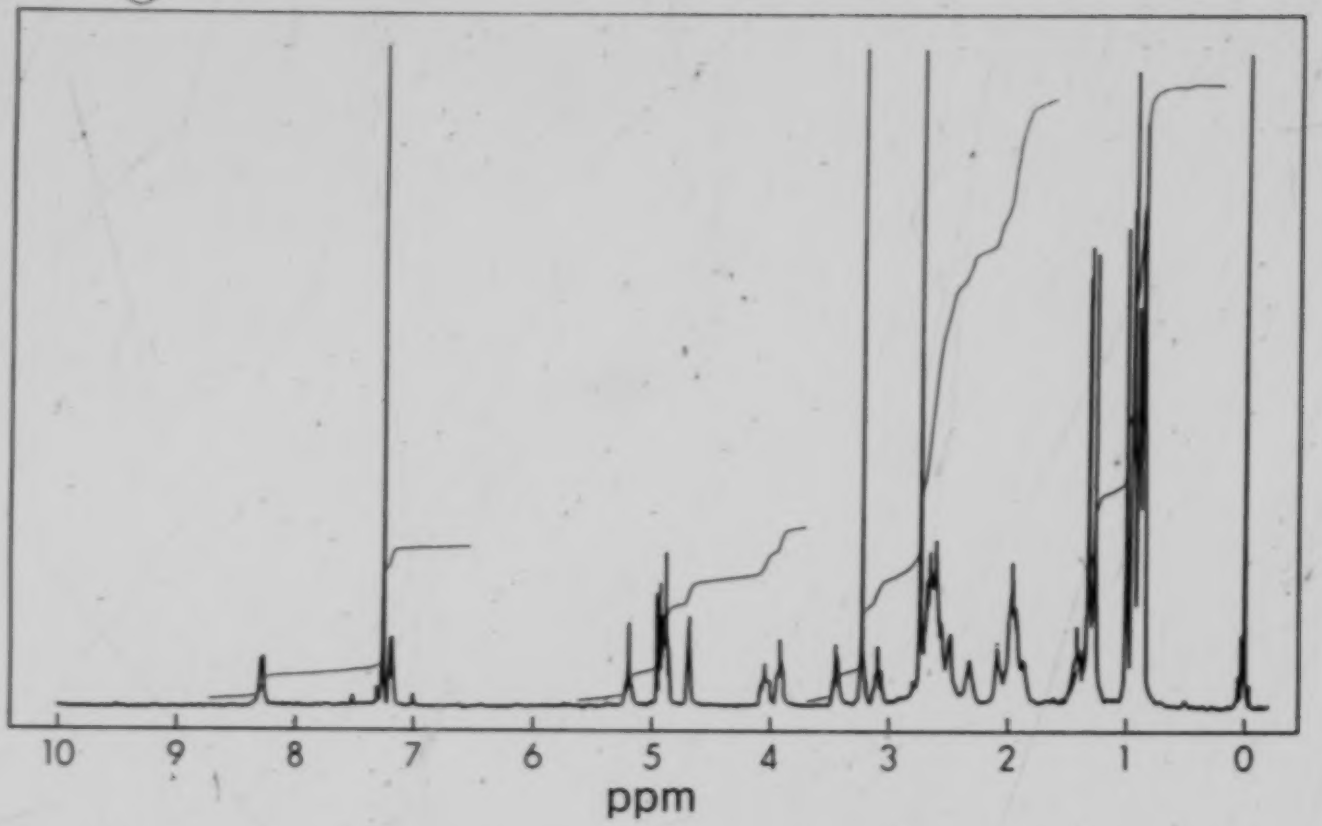

a
b
c
d
0
10
20
30
40
50
Elution Time (min)
A_{235}

Fig. II-2. A 400 MH_z proton NMR spectrum of *Alternaria brassicae*-toxin in $CDCl_3$. HPLC-purified toxin preparation was flash evaporated to dryness and dissolved in $CDCl_3$. It shows two protons on nitrogen at 7.17 and 8.28 ppm, two *N*-methyl protons at 2.74 and 3.23 ppm, five α-carbon protons in the region between 4.66 and 5.23 ppm, and seven methyl groups attached to carbon in the region between 0.84 and 1.35ppm. Other regions of the spectrum contain appropriate number of protons at positions consistent with the structure of Destruxin B. The peak at 7.25 ppm is $CDCl_3$.


10
9
8
7
6
5
4
3
2
1
0
ppm

Reactions of brassicas to *Alternaria brassicae* and the toxin

Reaction of brassicas to *A. brassicae* ranged from very susceptible to almost resistant [Fig. II-3(a) to II-3(c)]. A definite gradient of symptom severities was observed, and the order of decreasing susceptibility was *B. nigra*, *B. campestris* var. *yellow sarson* and var. *toria* > *B. juncea* cv. Lethbridge 22A > *B. campestris* cv. Candle, *B. napus* cv. Altex and *B. hirta* cv. Gisilba > *B. campestris* ssp. *rapifera* (Table II-1). This order of susceptibility matched in general the one mentioned by Dueck and Degenhardt (1975). Similarly, toxin-treated brassicas also developed symptoms of different severities [Figs. II-4(a) to II-4(c)]. The most susceptible hosts *B. nigra*, *B. campestris* cv. Yellow Sarson and cv. Toria showed severe symptoms with 240 and 120 μg/ml, and mild symptoms with 60 μg/ml of the toxin. *Brassica nigra* also developed visible chlorosis with 30 μg/ml of the toxin. The least susceptible host *B. campestris* ssp. *rapifera* did not show any symptoms even with 240 μg/ml of the toxin. Other hosts developed symptoms only with 240 and 120 μg/ml of the toxin, and the symptoms developed were mild except in the case of *B. juncea* which showed severe symptoms with 240 μg/ml of the toxin. None of the hosts showed any symptoms with 15 μg/ml of the toxin. Based on the severity of the symptoms, and differences in minimum concentration of the toxin required to cause visible chlorosis and necrosis, the order of sensitivity was *B. nigra*, *B. campestris* var. *yellow sarson* and var. *toria* > *B. juncea* cv. Lethbridge 22A > and *B. campestris* cv. Candle > *B. napus* cv. Altex and *B. hirta* cv. Gisilba > *B. campestris* ssp. *rapifera* (Table II-1). The order of sensitivity of brassicas to the toxin was similar to their order of susceptibility to *A. brassicae*. The results indicated that the toxin was able to distinguish susceptibility differences among *Brassica* species and also within the species *B. campestris*. Both inoculations with *A. brassicae* and treatments with the toxin indicated that *B. campestris* var. *yellow sarson* and var. *toria* are much more susceptible than *B. campestris* cv. Candle.

Fig. II-3. Symptoms caused by *Alternaria brassicae* on detached and scratched leaves of (a) *Brassica nigra*, (b) *B. juncea*, and (c) *B. campestris* ssp. *rapifera*. Inoculations with the fungus were carried out by placing a 50 µl drop of spore suspension (5 x 10^4 spores/ml) on the scratched area of the leaf. Leaves were incubated for 72 h on a moist filter paper in a Petri dish and kept at room temperature (about 22°C) under continuous illumination (about 560 lux).

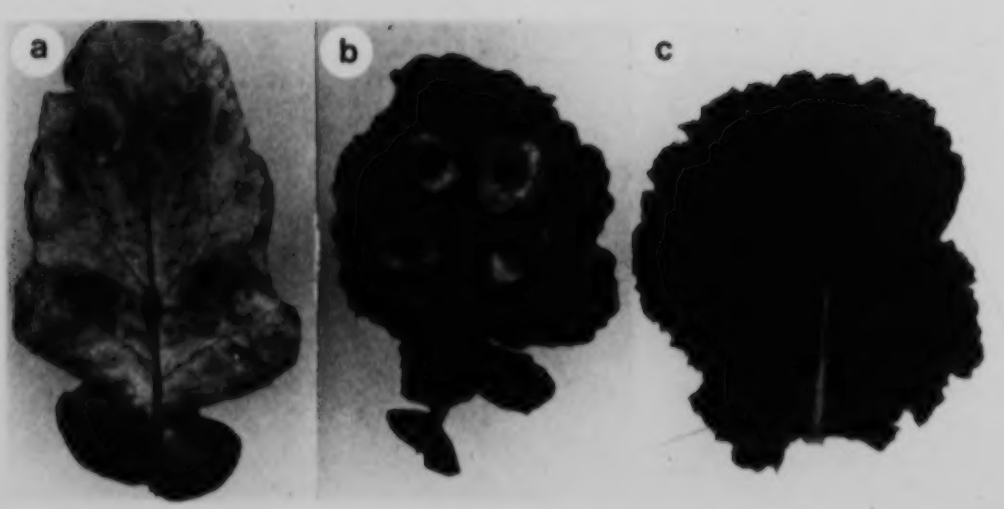

a
b
c

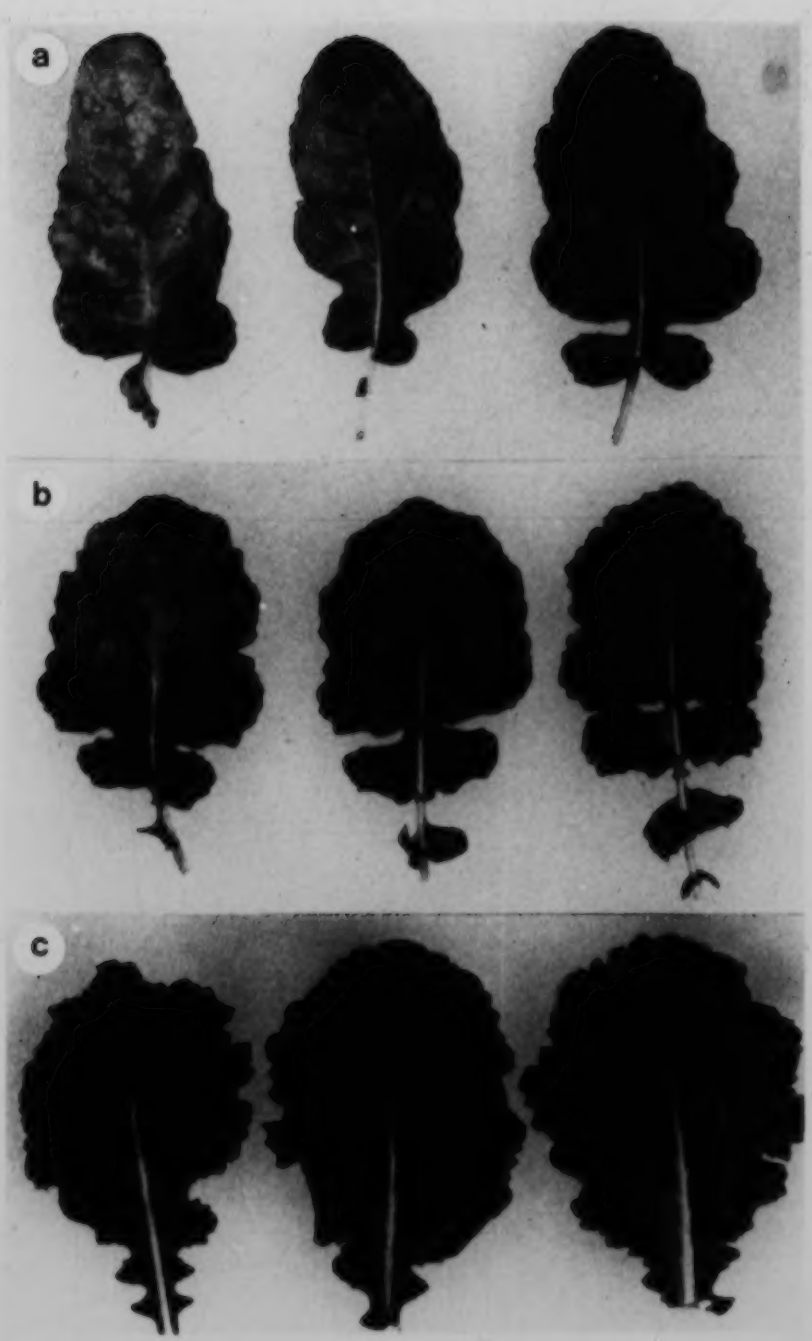
Table II-1. Symptoms produced by *Alternaria brassicae* and *A. brassicae*-toxin on the hosts and nonhosts of the fungus

Hosts/non-hosts	Symptom Intensity[1]	
	Fungus	Toxin[2]
B. nigra	+++	+++
B. campestris var. *yellow sarson*	+++	+++
B. campestris var. *toria*	+++	+++
B. juncea cv. *Lethbridge 22A*	++	++
B. campestris cv. Candle	+	+
B. napus cv. Altex	+	+
B. hirta cv. Gisilba	+	+
B. campestris ssp. *rapifera*	-	-
Avena sativa	-	-
Cucumis sativus	-	-
Hordeum vulgare	-	-
Linum usitatissimum	-	-
Lycopersicon esculentum	-	-
Secale cereale	-	-
Triticum aestivum	-	-
Vigna unguiculata	-	-
Zea mays	-	-

[1]Symbols: +++ = very severe, ++ = moderate, + = mild, - = no symptoms.

[2]Based upon severity of symptoms expressed in the Brassicas after 72 h when treated with 20 µl of 240, 120, 60, 30, and 15 µg/ml of the toxin and minimum concentration required for visible chlorosis development. The reactions of the non-hosts were recorded after 72 h of feeding 20 µl of 300 µg/ml toxin solution through the vascular system.

Fig. II-4. Responses of detached and scratched leaves of (a) *Brassica nigra*, (b) *B. juncea*, and (c) *B. campestris* ssp. *rapifera* to 240, 120, 60, 30 and 15 μg/ml of *Alternaria brassicae*-toxin and undiluted control fraction. The toxin solution was applied by placing a 20 μl drop of the solution on the scratched area of the leaf. One toxin concentration was applied to each half-leaf. From left to right: the sequence of treatments was; 240, 120, 60, 30 and 15 μg/ml of the toxin, and undiluted control fraction. Leaves were incubated for 72 h on a moist filter paper in a Petri dish, kept at room temperature (about 22°C) under continuous illumination (about 560 lux).


a
b
c

Reactions of nonhosts of *Alternaria brassicae* to the toxin

The nonhosts treated with the toxin did not develop any chlorosis and necrosis (Table II-1), whereas *B. campestris* var. *yellow sarson*, used as a control in this experiment, developed chlorosis and necrosis both with 300 and 30 μg/ml of toxin. The results suggest that a plant species which is not a host to *A. brassicae* is also not visually affected by the toxin produced by it.

Comparison of the effects of destruxin B and the toxin on *Brassica nigra* and *Brassica campestris* ssp. *rapifera*

Neither destruxin B nor the toxin caused any symptoms on *B. campestris* ssp. *rapifera*, but similar concentrations of destruxin B and the toxin caused symptoms of similar severities on *B. nigra* [Figs. II-5(a) and II-5(b)].

D. Discussion

An earlier study (Degenhardt, 1978) indicated that *A. brassicae* produced two groups of toxic compounds *in vitro*. The final preparations used to study the nature of these compounds were described as being semipurified. The chromatographic purification procedure described in the present study yielded only one toxin from culture filtrates of the fungus. During this purification procedure all columns were eluted exhaustively and all fractions showing toxic activity were pooled together for the next purification step. Activated charcoal was sequentially washed with two solvents, ethyl acetate and water-saturated *n*-butanol. Both solvent fractions were toxic suggesting that there may be two toxins. However, both showed identical amino acid composition, molecular weight and retention time on HPLC. Because of high resolution of HPLC, identical retention time provide *prima-facie* evidence that the two fractions contain the same toxin (Walton and Earle, 1984b). The reason for the differential elution pattern of the toxin from charcoal is not clearly known at this time. However, it could be that the toxin desorbed with water-saturated *n*-butanol is the residual toxin after ethyl acetate washings, which co-elutes with other materials present in the preparation. The butanol fraction took a long time to

Fig. II-5. Responses of detached and scratched leaves obtained from 5-week-old plants of (a) *Brassica nigra*, and (b) *B. campestris* ssp. *rapifera* to 120, 30, 7.5 and 0.0 μg/ml of destruxin B and the toxin isolated during this study. Left and right half-leaves were treated with similar concentrations of destruxin B and the toxin, respectively. A 20-μl droplet of test solution was applied to each scratched area. From left to right: the sequence of treatment on leaves was; 120, 30, 7.5 and 0.0 μg/ml of destruxin B and the toxin. Leaves were incubated for 72 h on a moist filter paper in a Petri dish, kept at room temperature (about 22°C) under continuous illumination (about 560 lux).

a)
b)

flash evaporate to dryness, therefore in subsequent experiments, water-saturated *n*-butanol was not used to desorb this residual toxin from the charcoal. The HPLC elution profile reflects on purity of the toxin preparation obtained from the silicic acid column and later used for host-specificity studies.

Although a toxin preparation of high purity was obtained from the silicic acid column and Sep-Pak C_{18} cartridge, for chemical analyses, toxin eluted in the peak fraction from HPLC was used. Degenhardt (1978) reported that semi-purified toxic fractions from *A. brassicae* culture filtrates showed negative tests for carbohydrates and phenols, but a positive Lowry test for peptides or small proteins. In the present study, the ninhydrin test applied to the toxin was negative, but the hydrolysed toxin reacted positively with ninhydrin. Negative ninhydrin reaction with the unhydrolysed toxin indicated that there are no free amino acids in the preparation and that there are no unsubstituted amino groups present in the toxin. However, the positive ninhydrin reaction after acid hydrolysis suggested the presence of amine groups. Furthermore, amino acid analyses showed that these hydrolysates contain amino acids. The presence of amino acids coupled with negative ninhydrin reaction of the unhydrolysed toxin showed that the toxin is a cyclic peptide. Two other *Alternaria* toxins, AM-toxin I and II produced in culture by *A. alternata* (fr.) Keissler f.sp. *mali* are also reported to be cyclic depsipeptides (Ueno *et al.*, 1975).

Further analyses of chemical characteristics, such as molecular weight, amino acid composition, and NMR spectrum of the toxin, suggested that these properties are similar to those of destruxin B, a cyclic depsipeptide toxin isolated primarily by partitioning with organic solvents from culture filtrates of *A. brassicae*, in another study on metabolites produced by the fungus (Peña-Rodriguez, 1985). The structure of destruxin B has been reported (Peña-Rodriguez, 1985; Tamura *et al.*, 1964), and the coupling pattern of the ^1H NMR spectrum obtained in the present study matched with the expected coupling pattern of this structure. The HPLC retention time of destruxin B was identical to that of the toxin isolated during the present study and a 1:1 mixture of destruxin B and the toxin eluted as a single symmetrical peak at 26 min. Both destruxin B and the toxin showed similar biological activity. Neither destruxin B nor the toxin caused any symptoms on *B.*

campestris ssp. *rapifera*, but similar concentrations of destruxin B and the toxin caused symptoms of similar severities on *B. nigra*. The aforesaid evidence considered collectively indicated that the toxin isolated during this study is destruxin B. Destruxin B is thus a unique phytotoxin as it is also produced by *Metarhizium anisopliae*, a pathogen of insects (Pais *et al.*, 1981; Tamura *et al.*, 1964) but a non-pathogen of rapeseed (Williams, 1985).

Alternaria brassicae and the toxin produced by it caused symptoms of different severities on different brassicas. The symptoms ranged from severe chlorosis and necrosis to extremely mild chlorosis. The order of sensitivity of different brassicas to the toxin was similar to the order of susceptibility of these brassicas to *A. brassicae* observed in the present study and also matched in general to the order of susceptibility of brassicas reported earlier from a field study (Dueck and Degenhardt, 1975). The toxin was also able to distinguish susceptibility differences within a species. Both *A. brassicae* and the toxin caused severe symptoms on *B. campestris* var. *yellow sarson* and var. *toria*, and relatively mild symptoms on *B. campestris* cv. Candle. The toxin was unable to cause symptoms on nine plant genera which are nonhosts of *A. brassicae*, whereas even 10 times diluted toxin caused symptoms on *B. campestris* var. *yellow sarson*, a host of the fungus which was used as a positive control. Host-specific toxins show correlation between sensitivity to the toxin and varietal disease reaction (Daly and Knoche, 1982). In the present study, sensitivity of a plant to the toxin was correlated with its susceptibiltiy to *A. brassicae*, suggesting that the toxin is host-specific and is a factor in disease development. Additional proof of this could come from the study of wild non-pathogenic strains of *A. brassicae*, but so far no such strains are known to occur.

The minimum concentration of the toxin (15-30 μg/ml) required for causing visible chlorosis is higher than that of other host-specific toxins. The reason for this high minimum concentration may be the low sensitivity of the scratched leaf bioassay method used in this study. Minimum concentrations of 10-15 μg/ml were reported for leaf puncture bioassay of *Helminthosporium maydis* race T-toxin, whereas dark CO_2 fixation or mitochondrial respiration bioassays were 20-100 times more sensitive for detecting this toxin (Earle *et al.*, 1978). Therefore, it is very likely that alternative sensitive methods of

bioassay may be developed for detecting a lower concentration of *A. brassicae*-toxin.

To date no host-specific toxin has been reported from *A. brassicae*. Two non-specific toxic groups of compounds reported earlier from culture filtrates of *A. brassicae* were of much higher molecular weights (Group I above 5000, and Group II between 2000 and 5000) than those of the other *Alternaria* toxins (Degenhardt, 1978). The cause of non-specificity may be due to the use of semipurified preparations. Some non-specific response with the partially purified toxin was also observed in preliminary experiments during the present study (Bains and Tewari, 1985). It is of interest to note that of seven pathogenic, large-spored, long-beaked *Alternaria* species, only *A. brassicae* does not produce zinniol which is a non-selective phytotoxin (Cotty and Misaghi, 1984).

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III. Bioassay and mode of action of *Alternaria brassicae* toxin

A. Introduction

Bioassay is required for every facet of toxin research including detection, purification, mode of action, and selection for disease resistance. Bioassay should be sensitive, quantitative, reproducible, and easy to perform. Bioassays used for the toxic compounds produced in culture filtrates of *A. brassicae* (Berk.) Sacc. were based on symptom development in seedlings and intact plants, detached and attached leaves, and detached branch bearing pods of rapeseed (Ayer and Peña-Rodriguez, 1987; Degenhardt, 1978; Husain and Thakur, 1966). However, these bioassay methods have been reported to be less sensitive than the ones based on the effects of toxin on some physiological or biochemical processes, or on isolated cells and protoplasts (Gardner *et al.*, 1986; Scheffer and Livingston, 1980; Yoder, 1981; Yoder *et al.*, 1977). Except in one study (Ayer and Peña-Rodriguez, 1987), bioassay of toxic compound(s) produced by *A. brassicae* was done using crude culture filtrates or semipurified toxin preparations. For better understanding of the biological characteristics and mode of action of a toxin, it is important to use pure toxin in combination with a sensitive bioassay method. Although purification of *A. brassicae* toxin (destruxin B) was described in two concurrent studies (Ayer and Peña-Rodriguez, 1987; Chapter No.2 of this thesis), there is still a need for a sensitive bioassay method. There is also a need to locate the initial site of destruxin B action, because this information is prerequisite to understanding its mode of action.

The objectives of this study were (1) to develop a sensitive, fast, and simple bioassay method for destruxin B, and (2) to determine the initial site of destruxin B action.

B. Materials and Methods

Electrolyte leakage from leaf disks

Leaf disks from third or fourth leaves from six-week-old greenhouse grown *A. brassicae*-resistant plants of *B. campestris* L. ssp. *rapifera*, and *A. brassicae*-susceptible plants of *B. campestris* var. *yellow sarson* were used for electrolyte leakage studies. Preliminary experiments were done by a method modified from Damann *et al.* (1974). Six disks of one cm diameter from the leaves of *B. campestris* var. *yellow sarson* were vacuum infiltrated for 20 min with 100 μg/ml destruxin B in de-ionized distilled water or in de-ionized distilled water as a control treatment. After infiltration, the leaf disks were washed twice with and finally resuspended in 5 ml of de-ionized water in a 50 ml Erlenmeyer flask. The flasks were shaken at room temperature on a wrist action shaker at 100 strokes per min. Conductivity of the ambient solution was measured after 0, 2, 5, 10, 20, 40, and 60 min, and then after every 2 h up to 12 h of incubation. The conductivity was measured by withdrawing the solution in the conductivity cell (1 ml capacity) of a Radiometer CDM83 conductivity meter. After determining the conductivity, the solution from the conductivity cell was returned to the flask.

An electrolyte leakage study was also conducted by a method modified from Mayama *et al.* (1986). Detached leaves from the susceptible and the resistant plants were rinsed with de-ionized distilled water, and allowed to plasmolyse at room temperature. After 10 min, the lower epidermis was removed using a pair of forceps. Leaf disks of 1 cm diameter were cut with a cork borer, and six leaf disks were used for each treatment. The leaf disks were placed, with their epidermis stripped side down, in a watch glass containing 4 ml of test solution. The test solutions included 1, 10, and 100 μg/ml destruxin B in de-ionized distilled water, and de-ionized distilled water was a control. The watch glass in turn was placed in a Petri plate containing a moistened filter paper to prevent the test solution from drying. The Petri plate was kept on a laboratory bench under continuous fluorescent light. The electrolyte leakage measurements were done as described earlier after 0, 3, 24, and 48 h of incubation. The conductivity value for a particular treatment was obtained by subtracting from it the conductivity value in that treatment at 0 time. After

the 48 h measurement, the ambient solutions were collected and kept frozen for further analyses. There were three replicates and the experiment was repeated once.

The amount of total sugars in the ambient solutions was determined by the method of Avigard (1975), and the results were expressed in terms of D-glucose. The amino acid content of ambient solutions was determined by the procedure of Spies (1957). Determination of calcium, magnesium, potassium, sodium, phosphorus, and sulphur was done by vacuum inductively coupled plasma atomic emission spectrometry (ICP-AES).[3]

Isolation of *Brassica campestris* var. *yellow sarson*, and *Camelina sativa* leaf mesophyll protoplasts

The third or fourth leaf from four- to five-week-old greenhouse grown plants of *B. campestris* var. *yellow sarson*, and fully expanded leaves from four-week-old greenhouse grown plants of *Camelina sativa* (L.) Crantz were used for protoplast isolation. Since *C. sativa* possesses high resistance to *A. brassicae* (Conn *et al.*, 1988), it was substituted for *B. campestris* ssp. *rapifera* as the resistant host in some of the experiments. The procedure for protoplast isolation was similar to the one used by Bains *et al.* (1988). The leaves were surface sterilized with 70 percent ethanol, rinsed twice in double distilled water, and allowed to plasmolyse at room temperature for 10-15 min. The lower epidermis of the leaf was removed, and the leaf was placed, with its epidermis stripped side down, in a Petri plate containing an enzyme solution. The enzyme solution contained 2 percent cellulase Onozuka R-10 (Kinki Yakult Manufacturing Co.), 0.1 percent pectolyase Y23 (Kikkoman Shoyu Co.) in 0.6M aqueous mannitol, pH adjusted to 5.5 with KOH. The Petri plate was kept in a waterbath at 30°C and was swirled occasionally. Most of the cells released protoplasts within one h. The enzyme mixture containing the protoplasts was passed through a double layer of cheesecloth and the supernatant was centrifuged for two min at 100 g. The protoplasts were washed twice and finally suspended in 0.6M mannitol.

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[3]I wish to thank Mr. Y. Kalra of Northern Forestry Centre, Canadian Forestry Service, Edmonton, and Dr. M. Gray of Alberta Research Council for ICP-AES.

Treatment of protoplasts with destruxin B

Treatment of protoplasts with destruxin B was achieved by incubating them in either toxin containing 0.6M mannitol or toxin containing incubation medium. The incubation medium used was that of Aoki and Takebe (1969). The concentrations of the toxin used were 25, 50, and 100 μg/ml, and the control treatment was protoplasts in 0.6M mannitol or the incubation medium. Protoplasts were incubated at room temperature under continuous fluorescent illumination in 35mm plastic Petri plate sealed with parafilm. Each Petri plate contained 100 μl of the protoplast suspension of 1.5 to 2.0 x 10^5 protoplasts/ml. The effect of the toxin on protoplast survival was observed after 24 and 48 h of incubation. The protoplast suspension was stained with fluorescein diacetate (FDA) as described by Zilakh and Gressel (1978). The stock solution of FDA (5mg FDA/ml of acetone, stored at -20°C) was diluted 50 times with 0.6M mannitol or the incubation medium. One hundred μl of this solution were added immediately to 100 μl of the protoplast suspension. After 5 min of incubation at room temperature, the percentage of fluorescing protoplasts was determined using a Carl Zeiss fluorescence microscope equipped with exciter filters BG12 and BG3 and barrier filter 65, and one hundred protoplasts were counted in each replication. There were 3 replicates for each treatment and the experiment was repeated 2 times.

Incubation of *Brassica campestris* var. *yellow sarson* and *Camelina sativa* pollen with destruxin B

Pollen was collected from recently dehisced anthers of *B. campestris* var. *yellow sarson* and *C. sativa*. *Camelina sativa* was used as a resistant host because, as mentioned earlier, it possesses high resistance to *A. brassicae*, and *B. campestris* ssp. *rapifera* did not flower regularly under our greenhouse conditions. The pollen grains were germinated using the hanging drop technique of Darlington and LaCour (1960), in a slightly modified medium of Brewbaker and Kwack (1963), the concentration of sucrose being reduced to 12%, as at higher sucrose concentrations the pollen tubes appeared shrunken. Treatment of

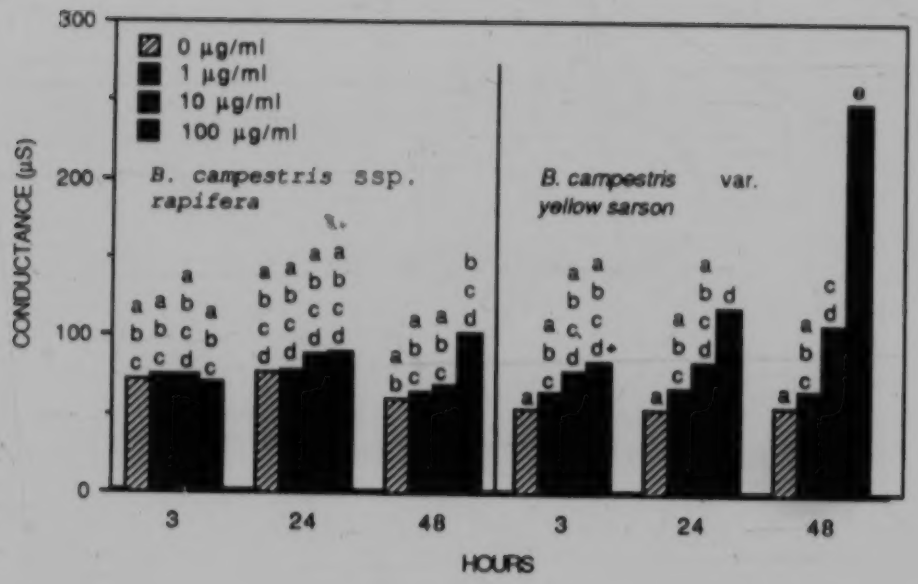
pollen with the toxin was achieved by suspending pollen in the medium containing the desired concentration of destruxin B. The toxin was used at concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 40.0, 60.0, 80.0, and 100.0 μg/ml, and the control was run in germination medium without the toxin. A 100 μl droplet of the medium, with or without the toxin, was placed on a microscope slide. An anther was removed from the flower with a pair of foreceps and gently touched with the surface of the droplet. The microscope slide was then inverted and kept on a watch glass, which in turn was kept in a Petri plate containing a moist filter paper. Incubation of pollen was done at room termperature under continuous fluorescent illumination on a laboratory bench. After 2 h of incubation, percent germination and pollen tube growth were recorded using a light microscope. Germination and pollen tube growth of *B. campestris* var. *yellow sarson* pollen incubated with 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 μg/ml destruxin B, and the incubation medium without the toxin, were also recorded after 30 min of incubation. Pollen was considered germinated when pollen tube length exceeded half the diameter of the pollen grain. Each treatment was replicated 3 times. The percent germination was obtained by counting germinated pollen grains in a total of 100 grains per hanging droplet, and the length of 30 pollen tubes was measured per hanging droplet using a calibrated ocular micrometer. The experiment was repeated twice. Pollen from the same batch of flowers was used for all the treatments of an experiment.

C. Results

Effect of destruxin B on leakage of electrolytes from leaf disks

Preliminary results indicated that destruxin B did not cause an increase in electrolyte leakage even after 12 h of incubation with the toxin. Therefore, electrolyte leakage measurements in subsequent experiments were taken up to 48 h of incubation. The results of the time-course study of electrolyte leakage both from susceptible and resistant leaf disks are presented in Figure III-1. Relative to 0 time, 3 h of incubation caused a

Fig. III-1. Effect of destruxin B on loss of electrolytes from leaf disks of the resistant host *Brassica campestris* ssp. *rapifera*, and the susceptible host *Brassica campestris* var. *yellow sarson*. Leaf disks were incubated with the toxin in a watch glass kept on a moistened filter paper in a Petri dish. Conductance of ambient solutions was monitored with a conductivity meter, and conductance value for a particular treatment was obtained by subtracting from it the conductance value at 0 time of the same treatment. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test). The experiment was repeated once with similar results.


300
200
100
0
CONDUCTANCE (µS)
0 µg/ml
1 µg/ml
10 µg/ml
100 µg/ml
B. campestris ssp. rapifera
B. campestris var. yellow sarson
3
24
48
3
24
48
HOURS

significant increase in the leakage of electrolytes, but the increase was observed in all the treatments of both resistant and susceptible tissues. This increase seems to be the result of leakage from cells that were damaged while removing the lower epidermis and cutting leaf disks. After 24 h of incubation, 100 μg/ml toxin-treated susceptible leaf disks showed significantly higher leakage than those of the control treatment, but the latter was not different from that caused by 10 μg/ml. All treatments given to resistant leaf disks caused similar amounts of leakage of electrolytes. After 48 h of incubation in 100 μg/ml, the toxin-treated susceptible leaf disks leaked significantly higher amount of electrolytes than all other treatments, and it was four times higher than that of the control treatment. The toxin at 10 μg/ml also caused significantly higher leakage of electrolytes than that of the control treatment, but it was similar to that caused by 1 μg/ml toxin.

Ambient solutions collected after 48 h of incubation were analyzed for amounts of total sugars, amino acids, calcium, magnesium, potassium, sodium, phosphorus, and sulphur (Table III-1). Susceptible leaf disks treated with 100 μg/ml of destruxin B released significantly higher amounts of all solutes and metabolites tested, except sodium, than that of control leaf disks. Whereas, 10 μg/ml caused higher amounts of leakage of only sugars and potassium compared with that of control leaf disks. No such differences were observed in the ambient solutions from resistant leaf disks.

Effect of destruxin B on survival of protoplasts

The method used for protoplast isolation yielded 80 to 90 percent intact protoplasts both from *C. sativa* and *B. campestris* var. *yellow sarson* leaves (Figs. III-2a and III-2b). Survival rates of protoplasts from susceptible and resistant hosts after 24 and 48 h of incubation with different concentrations of destruxin B were similar to those of their respective control treatments (Fig. III-3).

Table III-1. Effect of destruxin B on leakage of electrolytes and metabolites from *Brassica campestris* var. *yellow sarson* and *B. campestris* ssp. *rapifera* leaf disks.

Source of leaf disks	Destruxin B concentration (μg/ml)	Total Sugars (μM)	Amino acids (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	K^{+} (mM)	Na^{+} (mM)	P (mM)	S (mM)
B. campestris	0	29.66a	0.43ab	0.17a	0.06a	0.23a	0.15a	0.04a	0.12a
var.	1	44.73ab	0.59ab	0.28a	0.08ab	0.23a	0.10a	0.06a	0.22ab
yellow	10	61.18b	0.96b	0.34a	0.09ab	0.53b	0.12a	0.08ab	0.34ab
sarson	100	138.86c	1.86c	0.51b	0.13b	0.83c	0.17a	0.11b	0.49b
B.	0	26.73a	0.26a	0.28a	0.08ab	0.25a	0.13a	0.03a	0.09a
campstris	1	27.61a	0.25a	0.28a	0.08ab	0.29a	0.13a	0.04a	0.05a
ssp. *rapifera*	10	28.63a	0.28a	0.29a	0.09ab	0.34a	0.19a	0.04a	0.10a
	100	43.93ab	0.47ab	0.28a	0.09ab	0.47a	0.16a	0.06ab	0.17a

- Ambient solutions were collected after 48 h of incubation of leaf disks with destruxin B.
- Amounts of total sugars and amino acids were determined by the methods of Avigard (1975) and Spies (1957), respectively, whereas, Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, P, and S were determined by vacuum inductively coupled plasma atomic emission spectrometry (ICP-AES).
- Values are means of three replicates.
- Means sharing the same letter in a column are not significantly different at the 5% level (Duncan's new multiple range test).

Fig. III-2. Freshly isolated leaf mesophyll protoplasts of *Camelina sativa* (a) and *Brassica campestris* var *yellow sarson* (b) suspended in incubation medium. The bar represents 15μm.

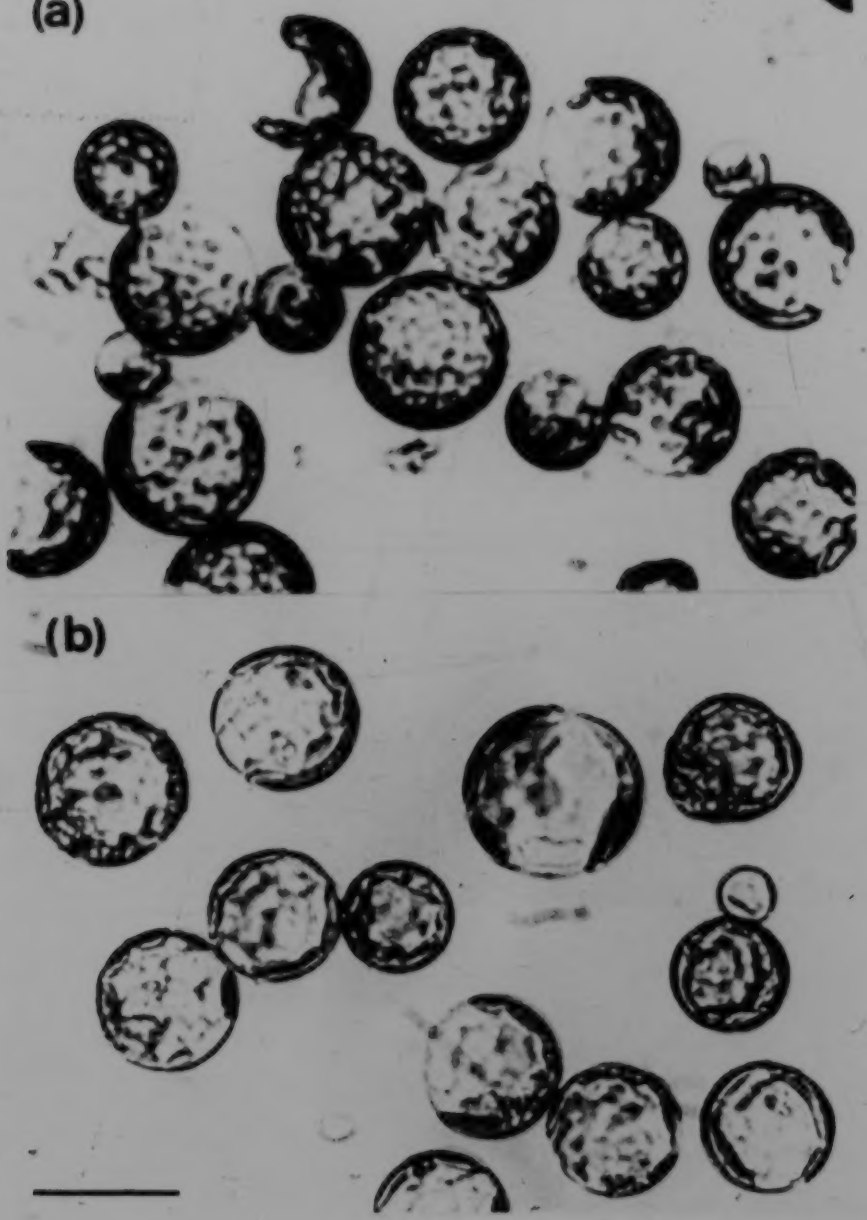
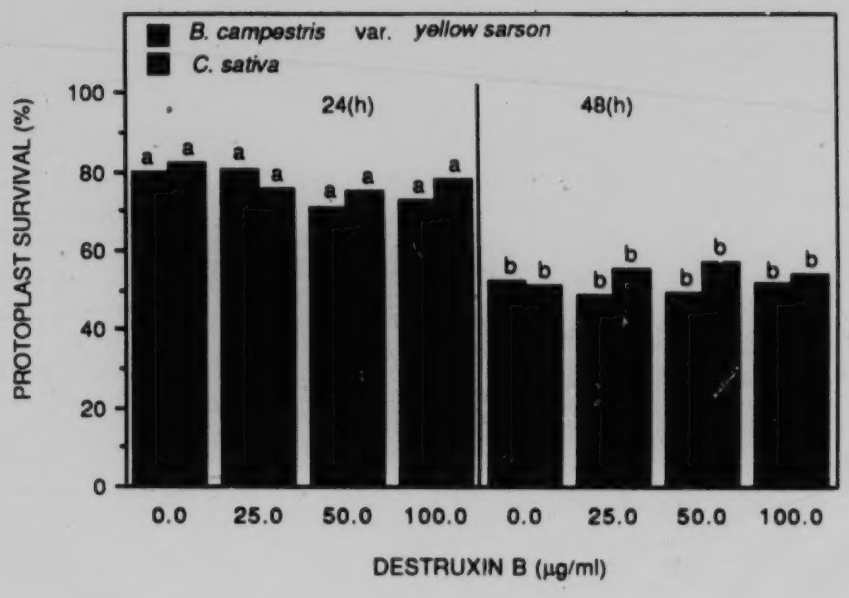

(a)
(b)

Fig. III-3. Effect of destruxin B on survival of leaf mesophyll protoplasts from the susceptible host *Brassica campestris* var. *yellow sarson* and the resistant host *Camelina sativa*. Enzymatically isolated protoplasts were treated with destruxin B by incubating them in toxin containing incubation medium. Survival rate of protoplasts was determined by observing fluorescein diacetate stained protoplasts with a fluorescence microscope. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test). The experiment was repeated 2 times with similar results.


B. campestris var. yellow sarson
C. sativa
24(h)
48(h)
PROTOPLAST SURVIVAL (%)
100
80
60
40
20
0
a a a a a a a a
b b b b b b b b
0.0 25.0 50.0 100.0 0.0 25.0 50.0 100.0
DESTRUXIN B (µg/ml)

Effect of destruxin B on pollen germination

The procedure and the medium used gave an excellent germination of pollen both from *B. campestris* var. *yellow sarson* and *C. sativa*. After 2 h of incubation in the medium, average germination of *B. campestris* var. *yellow sarson* pollen was 85 percent with an average pollen tube length of 65 μm and average germination of *C. sativa* pollen was 90 percent with an average pollen tube length of 170 μm (Figs. III-4a and III-4d). A differential effect of destruxin B was observed both on germination and tube growth of pollen from susceptible and resistant hosts (Figs. III-4a to III-4f, III-5 and III-6). Germination of *B. campestris* var. *yellow sarson* pollen was reduced with increasing toxin concentrations, and complete inhibition of germination took place at 12.5 μg/ml of the toxin (Figs. III-4a to III-4c and III-5). In contrast, 12.5 μg/ml of the toxin caused only 12 percent inhibition of germination of the resistant pollen from *C. sativa*, and a complete inhibition of germination of the pollen was not observed even at 100 μg/ml (the highest concentration tested) of the toxin (Figs. III-4d to III-4f and III-5). Although tube lengths of pollen from both *B. campestris* var. *yellow sarson* and *B. rapa* were affected, the effect on the pollen of former was much greater than that on the latter (Figs. III-4a to III-4f and III-6). Pollen tubes of the susceptible host did not grow further than a small protuberance (less than half the diameter of the pollen grain) when incubated for 2 h with 12.5 μg/ml of the toxin, whereas *C. sativa* pollen tubes of the resistant host grew to an appreciable length even when incubated for 2 h with 100 μg/ml of the toxin (the highest concentration tested).

Germination of *B. campestris* var. *yellow sarson* pollen was affected even after 30 min of incubation with destruxin B (Figs. III-7a to III-7b and III-8). The toxin at 2.5 μg/ml caused a significant reduction in germination, and a complete inhibition of germination was observed at 7.5 μg/ml. Similarly, pollen tube length was significantly reduced by 2.5 μg/ml of the toxin and only small protuberances were observed when pollen was incubated with 7.5 μg/ml of the toxin.

Fig. III-4. Effect of destruxin B on germination and tube growth of pollen from the susceptible host *Brassica campestris* var. *yellow sarson* (a, b, c), and the resistant host *Camelina sativa* (d, e, f). The pollen grains were incubated for 2 h in 100 μl hanging drops of germination medium containing 0 (a, d), 7.5 (b), 12.5 (c), 15 (e), and 100 (f) μg/ml of destruxin B. Bar represents 100 μm.

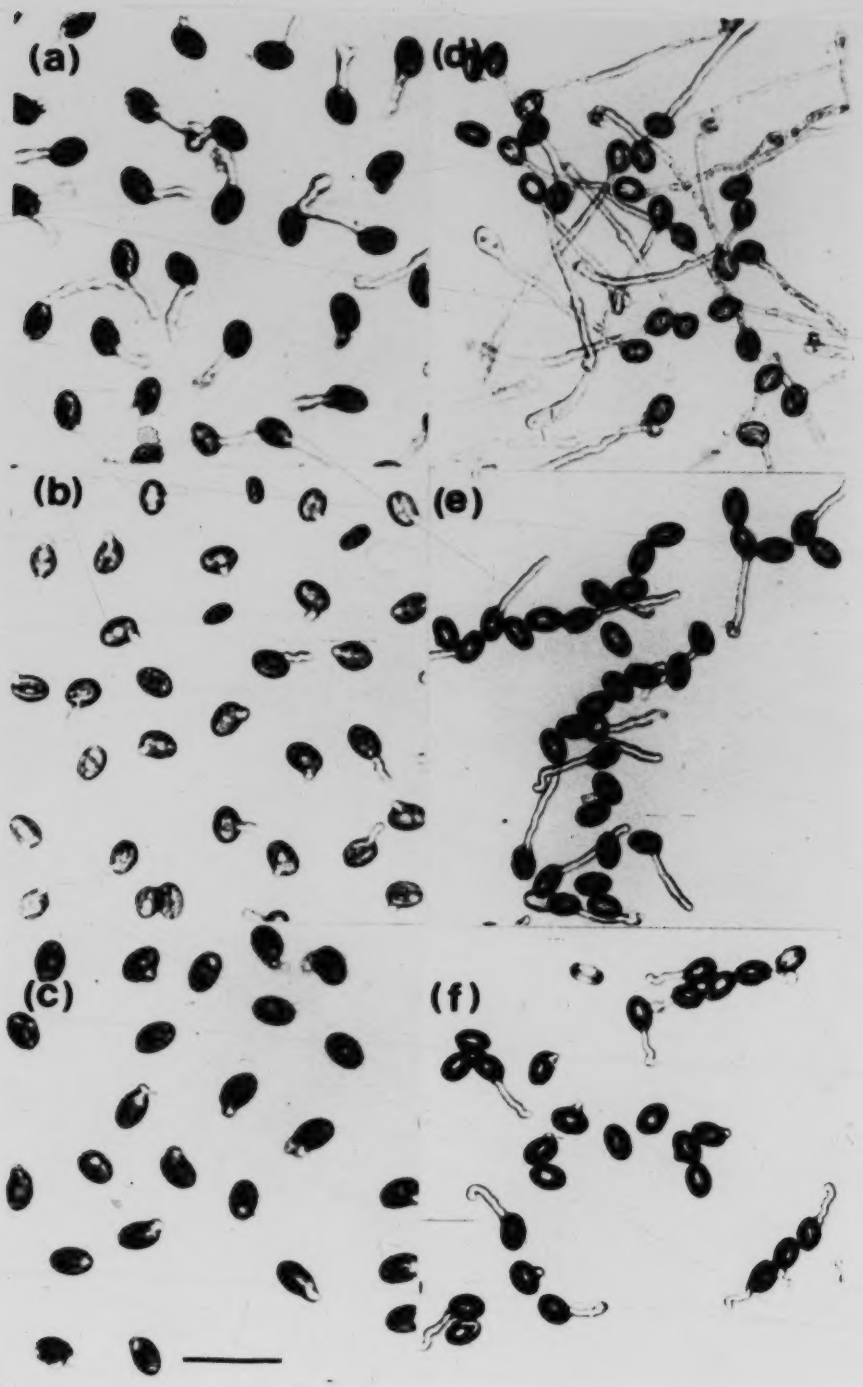

(a)
(b)
(c)
(d)
(e)
(f)

Fig. III-5. Effect of destruxin B on germination of pollen from the susceptible host *Brassica campestris* var. *yellow sarson* and the resistant host *Camelina sativa*. The pollen grains were incubated in 100 μl hanging drops of germination medium with or without destruxin B. Germination was recorded after 2 h of incubation at room temperature. A pollen grain was considered germinated when pollen tube length exceeded half the diameter of the pollen grain. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test). The experiment was repeated twice with similar results.

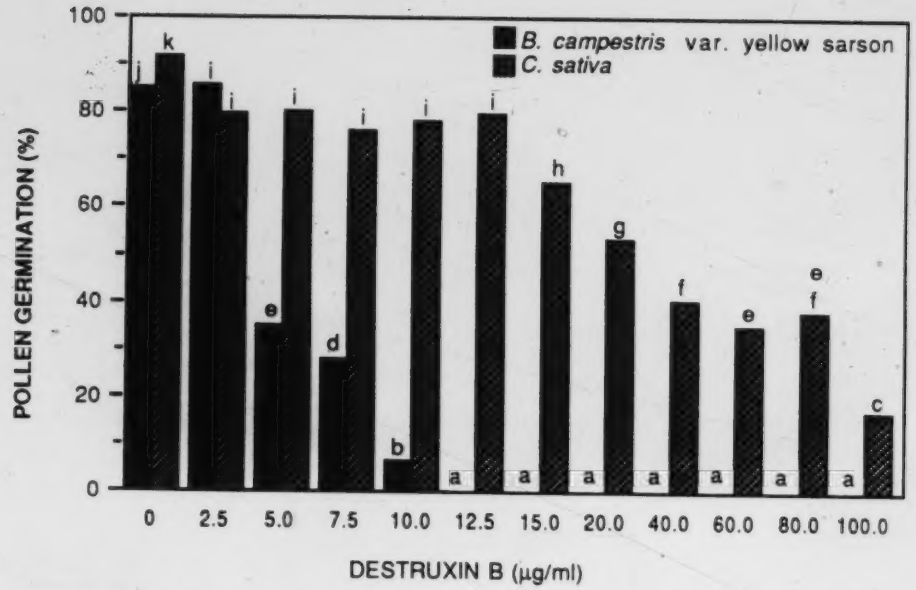

B. campestris var. yellow sarson
C. sativa
POLLEN GERMINATION (%)
100
80
60
40
20
0
0
2.5
5.0
7.5
10.0
12.5
15.0
20.0
40.0
60.0
80.0
100.0
DESTRUXIN B (µg/ml)

Fig. III-6. Effect of destruxin B on pollen tube length of the susceptible host *Brassica campestris* var. *yellow sarson* and the resistant host *Camelina sativa*. The pollen grains were incubated in 100 μl hanging drops of germination medium with or without destruxin B. After 2 h of incubation at room temperature, pollen tube lengths were measured using a calibrated ocular micrometer. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test). The experiment was repeated twice with similar results.

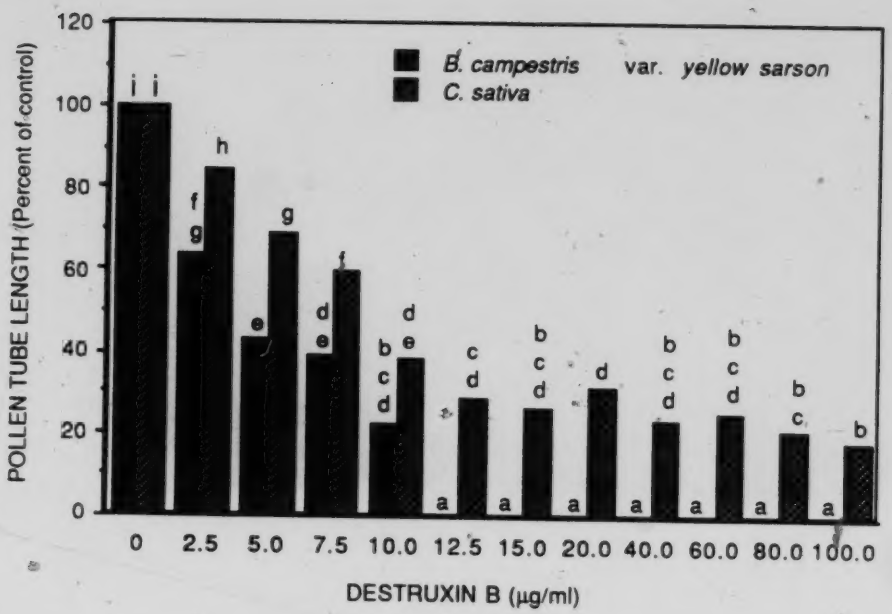

B. campestris var. yellow sarson
C. sativa
POLLEN TUBE LENGTH (Percent of control)
120
100
80
60
40
20
0
i i
h
f g
g
e
g
d e
f
b c d
d e
a
c d
a
b c d
a
d
a
b c d
a
b c d
a
b c
a
b
0
2.5
5.0
7.5
10.0
12.5
15.0
20.0
40.0
60.0
80.0
100.0
DESTRUXIN B (µg/ml)

Fig. III-7. Effect of destruxin B on germination of susceptible pollen from *Brassica campestris* var. *yellow sarson*. The pollen grains were incubated for 30 min in 100 μl hanging drops of germination medium (a), and germination medium containing 7.5 μg/ml destruxin B (b). Bar represents 100 μm.

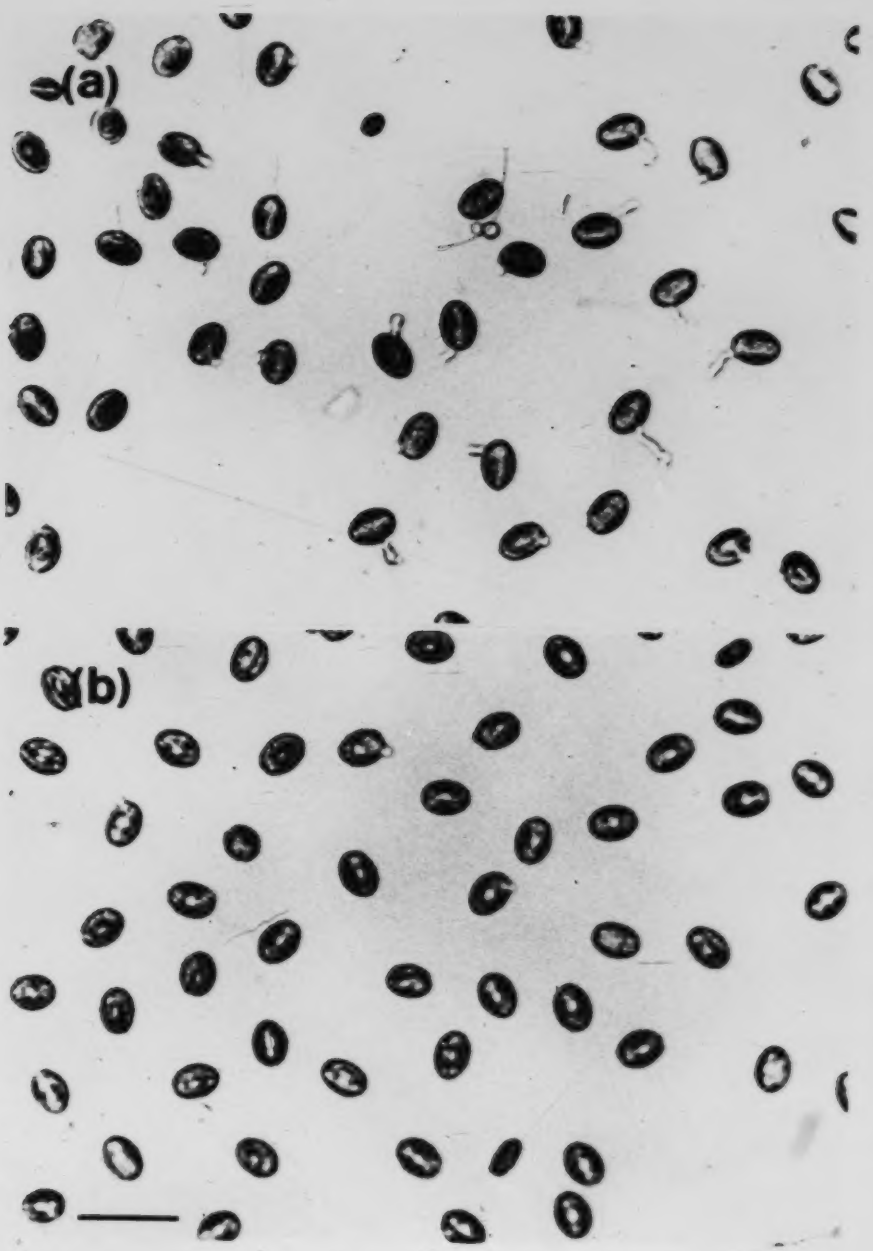
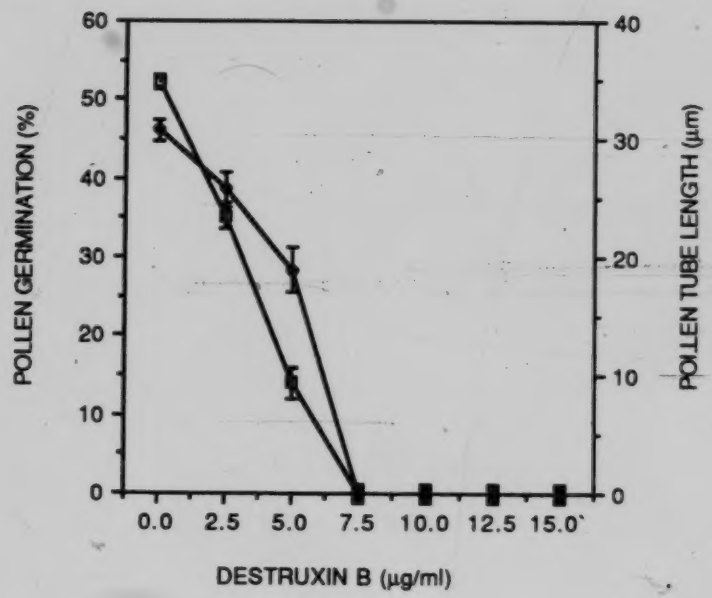

(a)
(b)

Fig. III-8. Effect of destruxin B on germination (·), and tube length (·) of susceptible pollen from *Brassica campestris* var. *yellow sarson*. The pollen was incubated in 100 μl hanging drops of germination medium with or without destruxin B. Germination and tube length of pollen were recorded after 30 min of incubation at room temperature. Pollen was considered germinated when pollen tube length exceeded half the diameter of the pollen grain. Pollen tube lengths were measured using a calibrated ocular micrometer. Bars indicate standard deviation. The experiment was repeated twice with similar results.


POLLEN GERMINATION (%)
POLLEN TUBE LENGTH (µm)
DESTRUXIN B (µg/ml)
0
10
20
30
40
50
60
0.0
2.5
5.0
7.5
10.0
12.5
15.0

D. Discussion

Four bioassay methods for destruxin B were compared for their sensitivity, rapidity, and simplicity. The bioassays used were 1) symptom development on detached leaves, 2) electrolyte leakage from leaf disks, 3) protoplast viability, and 4) pollen germination. The procedure and results of the detached leaf bioassay were described in chapter number 2 of this thesis. All the bioassays tested differed in their sensitivity, speed, and technical complexity.

The limits of destruxin B detection by the detached leaf bioassay were between 15 and 30 μg/ml. The bioassay distinguished all two-fold toxin dilutions between 15 and 120 μg/ml, but could not distinguish between the control and 15μg/ml toxin treatments. The bioassay was sensitive enough to distinguish susceptibility differences among different brassicas as well as within the species *B. campestris*. Bioassay methods based on symptom development on seedlings, intact plants, detached and attached leaves, and detached branches bearing pods have also been used to detect toxic compounds produced in culture by *A. brassicae* (Ayer and Peña-Rodriguez, 1987; Degenhardt, 1978; Husain and Thakur, 1966). However, these bioassay methods can not be compared with the bioassay methods used in this study because Degenhardt (1978) and Husain and Thakur (1966) used crude culture filtrates or semi-purified toxic preparations, and Ayer and Peña-Rodriguez (1987) used the detached leaf bioassay method only to detect the presence or absence of destruxin B. The leaf bioassay method was easy to perform but required about 48 h to obtain the results. Yoder *et al.* (1977) also indicated that the leaf lesion bioassay was easiest to perform but was much less sensitive than dark CO_2 fixation and root growth bioassay methods for HMT-toxin. Although the leaf bioassay is neither very sensitive nor very quantitative, but is very useful for many different types of experiments (Scheffer, 1976), and is still very widely used (Clouse *et al.*, 1985; Gardner *et al.*, 1985, 1986; Namiki *et al.*, 1986; Otani *et al.*, 1985; Vidhyasekaran *et al.*, 1986). In fact, it is generally the first bioassay method for demonstrating the role of toxin in disease development.

The electrolyte leakage bioassay, performed using leaf disks from the susceptible host *B. campestris* var. *yellow sarson*, could distinguish between control and 100 μg/ml toxin treatments both after 24 and 48 h of incubation of the leaf disks, and between control and 10 μg/ml of the toxin after 48 h of incubation of the leaf disks with the respective test solutions. However, the assay was unable to differentiate between 100 and 10 μg/ml of the toxin after 24 h of incubation, and between 10 and 1 μg/ml of the toxin after 48 h of incubation. The electrolyte leakage bioassay for destruxin B did not possess its characteristic feature of rapid electrolyte loss and was neither sensitive nor quantitative for the toxin. The results suggested that electrolyte leakage was not the initial physiological effect of the toxin, and is not a suitable bioassay technique for destruxin B. A delay of 30 to 40 h in electrolyte leakage was observed from *A. alternata* (Fr.) Keissler f. sp. *lycopersici* toxin (AAL-toxin)-treated susceptible tissue (Kohmoto *et al.*, 1982; McFarland and Gilchrist, 1982), and no electrolyte leakage was observed within 5-6 h after *Helminthosporium carbonum* Ullstrup toxin (HC-toxin) treatment of susceptible tissue (Scheffer and Livingston, 1984; Yoder and Scheffer, 1973). Electrolyte leakage bioassay was also reported to be less sensitive than root growth and dark CO_2 fixation bioassays for *H. maydis* Nisikado & Miyake race T toxin (HMT-toxin) (Bhullar *et al.*, 1975), but more sensitive than the leaf bioassay for *H. sacchari* (Van Breda de Haan) Butler toxin (HS-toxin) (Scheffer and Livingston, 1980). Based on the information available at the time, Scheffer (1976) commented that electrolyte leakage bioassay is of similar sensitivity but is faster than root growth bioassay. Contrary to the results obtained in this study, many host-specific toxins cause a rapid leakage of electrolytes, and the method has proven to be an excellent bioassay technique for those toxins (Damann *et al.*, 1974; Kohmoto *et al.*, 1976; Livingston and Scheffer, 1980, 1984; Otani *et al.*, 1975; Samaddar and Scheffer, 1971; Tsuge *et al.*, 1985; Yamamoto *et al.*, 1984).

Leaf mesophyll protoplasts from susceptible host that had been treated with 100 μg/ml of destruxin B showed a survival rate similar to that of the control treatment, whereas a treatment with 30 μg/ml toxin caused chlorosis in leaves from the same host.

The results suggested that the protoplast bioassay can not be used for destruxin B. Contrary to the results of this study, protoplast bioassay has been described to be a sensitive bioassay method (Walton and Earle, 1984b), and the bioassay has been used to monitor the effect of HMT-toxin (Aldrich *et al.*, 1977; Earle *et al.*, 1978; Gregory *et al.*, 1980; Pelcher *et al.*, 1975; Walton *et al.*, 1979), and *H. victoriae* Meehan & Murphy produced (HV-toxin) (Briggs *et al.*, 1984; Hawes, 1983a).

The results showed that destruxin B affected germination of susceptible pollen. Germination and tube length of the pollen were significantly reduced after 30 min of incubation with 2.5 μg/ml of the toxin, and no germination was observed after 30 min of incubation with 7.5 μg/ml of the toxin. The results from different studies taken together suggested that the pollen germination bioassay is a very sensitive, quantitative, fast, and easy bioassay for destruxin B.

Bioassay methods based on intact plants or tissues face the problems of uneven distribution of the toxin, and differential sensitivities of different cell types to the toxin (Hawes, 1983b; York *et al.*, 1980). The pollen germination bioassay avoids both of these problems. The pollen germination bioassay has not been widely used. It was used for bioassaying HMT-toxin (Laughnan and Gabay, 1973), and culture filtrates of *A. brassicicola* (Schw.) Wiltshire (Hodgkin and MacDonald, 1986). It was suggested to be more sensitive than seedling and root growth bioassay methods for HMT-toxin (Yoder *et al.*, 1977). Hodgkin and MacDonald (1986) also indicated that the assay is simple, fast, sensitive, and requires only a small amount of the compound.

Rapid electrolyte leakage observed in many susceptible host-toxin systems has been considered to indicate that the initial site of toxin action is on the plasma membrane (Kohmoto *et al.*, 1987; Maekawa, 1984; Nishimura and Kohmoto, 1983; Scheffer and Livingston, 1984). Therefore, observed absence of rapid electrolyte leakage indicates that the plasma membrane may not be the initial site of destruxin B action. However, delayed electrolyte leakage and increased amounts of all metabolites and electrolytes, except sodium, tested in the ambient solutions collected after 48 h of incubation of susceptible leaf disks

with 100 μg/ml destruxin B suggest that the toxin eventually causes changes or disruption of the plasma membrane. Similar results obtained earlier lead to similar conclusions for AAL-, and HC- toxins (Kohmoto *et al.*, 1982; McFarland and Gilchrist, 1982; Scheffer and Livingston, 1984; Yoder and Scheffer, 1973). The initial site of AAL-toxin action is not the plasma membrane, but the enzyme aspartate transcarbamoylase (Gilchrist, 1983; McFarland and Gilchrist, 1981), and as yet there is no proposed mechanism of HC-toxin action (Daly, 1981; Scheffer and Livingston, 1984).

Nonsensitivity of *B. campestris* var. *yellow sarson* protoplasts to the toxin suggested that the protoplasts do not have the receptor site for destruxin B, whereas the sensitivity of both the intact leaf tissue and pollen from the same host indicated that the sites may be present in them. These effects also suggested that the cell wall or periplasmic space may be the intial site of destruxin B binding. Similar studies were also conducted to understand the role of the cell wall in HV-toxin action (Briggs *et al.*, 1984; Hawes, 1983a). Both these studies showed that the susceptible oat cells with or without cell walls showed similar rates of cell death. It was concluded that the cell wall is not the site of lethal effect of HV-toxin.

Nonsensitivity of the protoplasts, observed in this study, does not appear to be due to changes that may have been caused by cell wall degrading enzymes and/or hypertonicity of the medium in which they were suspended, because protoplasts have been shown to be sensitive to HMT-toxin (Earle *et al.*, 1978; Gregory *et al.*, 1980; York *et al.*, 1980), and HV-toxin (Briggs *et al.*, 1984; Hawes, 1983a). Cell wall area has also been proposed to be the site of primary action of some other toxins. Based on ultrastructural effects of toxins on susceptible and resistant tissues, it was proposed that the plasma membrane/cell wall domain in the cell is the initial site of action of ACTG-toxins produced by *A. citri* Ellis & Pierce affecting rough lemon (now known as *A. alternata* rough lemon pathotype) (Kohmoto *et al.*, 1987), AF-toxins I and II, AK-toxins, and AM-toxin I, produced by strawberry, Japanese pear, and apple pathotpes of *A. alternata*, respectively (Park *et al.*, 1981). However, Kohmoto *et al.* (1987) indicated that evidence from physiological and

ultrastructural studies taken together suggest that AF-toxins I and II, AK-toxin, and AM-toxin I are most likely to disturb the membrane integrity of the susceptible cells. Studies of ultrastructural effects of destruxin B and specific binding of the toxin to this region may provide further support to the proposed site of initial destruxin B action. It is of interest to note that, as revealed by electron microscopy, the plasma membrane is the first part of the cell that is affected during pathogenesis by *A. brassicae* on canola (Tewari, 1983).

All cultivars of rapeseed are susceptible to *A. brassicae*, although those of Argentine rapeseed are less susceptible than those of Polish rapeseed (Conn, 1986; Skoropad and Tewari, 1977; Tewari and Skoropad, 1976; Tewari, 1985; Tewari *et al.*, 1988). High degrees of resistance to *A. brassicae* have been identified in some wild crucifers, including *C. sativa* and *Capsella bursa-pastoris* (L.) Medic, and moderate degrees in an accession of turnip (Chapter No. 2 of this thesis; Conn *et al.*, 1988; Tewari *et al.*, 1987). Somaclonal variation produced in cultured cells and tissues in combination with the toxin provide a good system for selection of new disease resistant genotypes of plant species (Brettell and Ingram, 1979; Earle, 1978; Ingram and MacDonald, 1986; Larkin and Scowcroft, 1981; Scowcroft *et al.*, 1983; Yoder, 1981). The results of this study indicated that pollen from susceptible and resistant plants showed differential germination responses to the toxin. Germination and tube legth of susceptible pollen were completely inhibited after 2 h of treatment with 12.5 μg/ml of destruxin B, whereas resistant pollen germinated and its pollen tube elongated even at 100 μg/ml of the toxin (the highest concentration tested). The results also indicated that qualitatively both pollen and intact leaf tissue are sensitive to the same compound. Therefore, *in vitro* pollen culture in the presence of destruxin B might provide an excellent system for *in vitro* selection for *A. brassicae* resistance. A similar system, but using culture filtrate extract, instead of purified toxin, has also been suggested for *A. brassicicola* resistance selection (Hodgkin and MacDonald, 1986). Use of toxin for *in vitro* selection for disease resistance has been suggested to be one of the most successful strategies (Ingram and MacDonald, 1986). There are several reports indicating various degrees of success in *in*

vitro selection for disease resistance using toxins (Brettell *et al.*, 1980; Gengenbach and Green, 1975; Gengenbach *et al.*, 1977; Rines and Luke, 1985; Sacristán, 1982, 1985; Thanutong *et al.*, 1983), and culture filtrates (Behnke, 1979, 1980; De March *et al.*, 1986; Dutrecq, 1981; Hartman, 1984; MacDonald and Ingram, 1986).

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IV. *Alternaria brassicae* toxin: a herbicide

A. Introduction

Fewer than 250 species of weeds in the world cause billions of dollars of damage annually (Holmes *et al.*, 1977). Economically important weeds belonging to the family Cruciferae occur in all provinces in Canada (Frankton and Mulligan, 1974). *Thlapsi arvense* L. (stinkweed) is one of the most serious agricultural weeds in the prairie provinces. Over a 5 year period, a 16% infestation of stinkweed reduced wheat yields by an average of 36%, while a 61% infestation reduced the yields by an average of 51% over hand-weeded controls (Best and McIntyre, 1975). *Sinapis arvensis* L. (Wild mustard) is another economically important weed belonging to Cruciferae. It also occurs in all provinces of Canada and is a serious weed on cultivated land in the prairies, particularly in cereal crops (Mulligan and Bailey, 1975).

Naturally occurring compounds are becoming increasingly more popular than synthetic chemicals as herbicides. Biologically produced compounds are easily degraded or detoxified in the environment as compared with the synthetically produced herbicides that are mostly halogenated hydrocarbons (Duke, 1986b). Although there are many examples of the usage of microorganisms as biological control agents of weeds (Anonymous, 1986a; Hasan, 1974; Julien, 1982; Templeton *et al.*, 1978), microbially produced phytotoxins have some definite advantages over living organisms as weed control agents. The major advantages are that these compounds do not pose any danger of spreading disease to non-target plants, and their effect is less dependent on environmental conditions (Duke, 1986a).

One of the conclusions from work presented in chapter number two of this thesis was that *Alternaria brassicae* (Berk.) Sacc. toxin (destruxin B) shows a host-specific activity. Hosts of *A. brassicae* include large number of species belonging to the family Cruciferae (Ellis, 1971), and cereals are known to be nonhosts of the fungus. This study was undertaken to investigate if destruxin B can be used as a herbicide against cruciferous

weeds in cereals.

B. Materials and Methods

Plant materials

All plants used in this study were grown from seeds. Before seeding, the seeds of *Capsella bursa-pastoris* (L.) Medic. (Shepherd's purse), *Erysimum cheiranthoides* L. (Wormseed mustard), *Lepidium densiflorum* Schrad. (peppergrass), *Neslia paniculata* (L.) Desv. (ball mustard), *Sinapis arvenis* L. (wild mustard), and *Thlapsi arvense* L. (stinkweed) were treated overnight with 1,000 ppm gibberellic acid, except the seeds of *Avena sativa* L. cv. Grizzly (oats), *Brassica campestris* L. cv. Tobin, *B. napus* L. cv. Westar (Canola), *Hordeum vulgare* L. cv. Galt (barley), *Secale cereale* L. cv. Muskateer (Rye), and *Triticum aestivum* L. cv. Neepawa (wheat). Seeds of individual species were sown in a row in a 26 x 26 x 6 cm or 52 x 26 x 6 cm plastic tray filled with moistened vermiculite. The number of rows in each tray varied based on the plant species being tested. The rows were approximately seven cm apart from each other. The tray was submerged in a reservoir tray containing half-strength Hoagland's solution modified by adding iron chelate to contain 1.5 g/ml iron. The trays were kept in a walk-in growth room at 23/19 C day/night termperature with an 18 h photoperiod and a photon flux density of 18,000 lux.

Plants of canola, peppergrass, shepherd's purse, stinkweed, wild mustard, barley, oats, rye, and wheat were also grown in the field at The Edmonton Research Station, University of Alberta. Seeds were treated as described earlier and seeds of each species were sown in a row. The rows were 60 cm long and the inter-row spacing was approximately 7 cm. The rows of weed species were interspersed among rows of cereal species. A guard row of wheat was sown all around the plot. A total of 6 such plots were seeded.

Application of destruxin B

A chromatographic sprayer was used to spray the growth room grown two week-old-plants with control or toxin solutions. The control solution contained 10% aqueous ethanol and 0.5% Tween 20, and the toxin solution contained destruxin B at a concentration of 8.0×10^{-4}M in the control solution. One 26 cm line of plants in a 26 x 26 x 6 cm or 52 x 26 x 6 cm tray received one ml of either solution. Three trays were sprayed with either solution and the effects of the toxin were noted after 5 days, and in a few instances again after 9-11 days of spraying. The experiment was repeated once.

Four field plots that showed good germination were selected for spraying. An atomizer was used for spraying. Each plot was divided breadth-wise into 2 equal halves, one half was sprayed with the toxin solution and the other half was sprayed with the control solution. The control and the toxin solutions were similar to the one used for the growth room study. Each half plot received 9 ml of either solution. The half plot being sprayed was enclosed in a three sided cardboard box to protect the surrounding plots from any spray drift. The reactions of the plants were noted after 6 days of spraying. Heights of 12 randomly selected plants were measured. For dry and fresh weight measurements, equal numbers of plants of a given species both from the control and the toxin treated half plots (5 for rye; 7 each for canola, wild mustard, wheat, barley, and oats; 15 for stinkweed and shepherd's purse; and 20 for peppergrass) were cut at the ground level and put in polyethylene bags. Plants were weighed for their fresh weights and then transferred to a brown paper bag and dried in an oven at 60°C. Dry weights were taken after 5 days of drying.

C. Results

The growth room-grown plants of all weed species and both cvs. of canola were affected by the toxin, but there was a difference in the severity of the symptoms developed. Five days after treatment, plants of ball mustard, shepherd's purse, stinkweed, wild mustard, and wormseed mustard showed very severe chlorosis and necrosis which often led

to the death of plants (Figs. IV-1 to IV-3). Canola cvs. Tobin and Westar showed severe chlorosis, necrosis, and growth suppression, whereas peppergrass developed moderate chlorosis and necrosis (Figs. IV-1 and IV-3). All plant species sprayed with the control solution, and toxin treated plants of barley, oats, rye, and wheat did not show any chlorosis or necrosis (Figs. IV-1 to IV-5).

After 11 days of spraying, plants of shepherd's purse, stinkweed, and wild mustard were completely necrotic and dead, whereas plants of canola cvs. Tobin and Westar, and wormseed mustard showed few green areas but their growth was markedly suppressed as compared to that of the control plants. After 9 days of spraying, barley, oats, rye, and wheat plants did not show any chlorosis or necrosis due to the toxin treatment.

In the field, plants treated with the toxin developed a range of reactions. Wild mustard and stinkweed revealed very severe chlorosis and necrosis that eventually lead to the death of most plants (Figs. IV-6 to IV-8). Canola cv. Westar developed severe chlorosis, necrosis, and growth suppression, peppergrass showed symptoms of intermediate severity, and shepherd's purse plants developed only mild chlorosis and necrosis (Fig. IV-6). Plants sprayed with the control solution as well as toxin-treated plants of barley, oats, rye, and wheat did not show any chlorosis or necrosis (Figs. IV-6 to IV-8). Heights, fresh weights, and dry weights of toxin treated canola cv. Westar, peppergrass, stinkweed, and wild mustard plants were significantly less than those of the control plants (Table IV-1). However, there were no significant differences in the heights, fresh weights, and dry weights of toxin-treated and control plants of barley, oats, rye, shepherd's purse, and wheat (Table IV-1).

Fig. IV-1. Growth room grown stinkweed, canola cvs. Westar and Tobin, peppergrass, and shepherd's purse (top to bottom rows) showing the effect of destruxin B. A chromatographic sprayer was used to spray two-week-old plants with control solution (left tray) or destruxin B solution (right tray). The control solution contained 10% aqueous ethanol and 0.5% tween 20, and the toxin solution contained destruxin B at a concentration of 8.0×10^{-4}M in the control solution. Each tray received 5 ml of either solution. Sprayed-plants were kept in the growth room and reactions of the plants were noted after 5 days of spraying.

Fig. IV-2. Growth room grown wild mustard (left row) and wormseed mustard (right row) showing the effect of destruxin B. Plants in the top and bottom trays were sprayed with destruxin B and control solutions, respectively. The protocol was identical to that of Fig. IV-1 except that each tray received 2 ml of either solution.

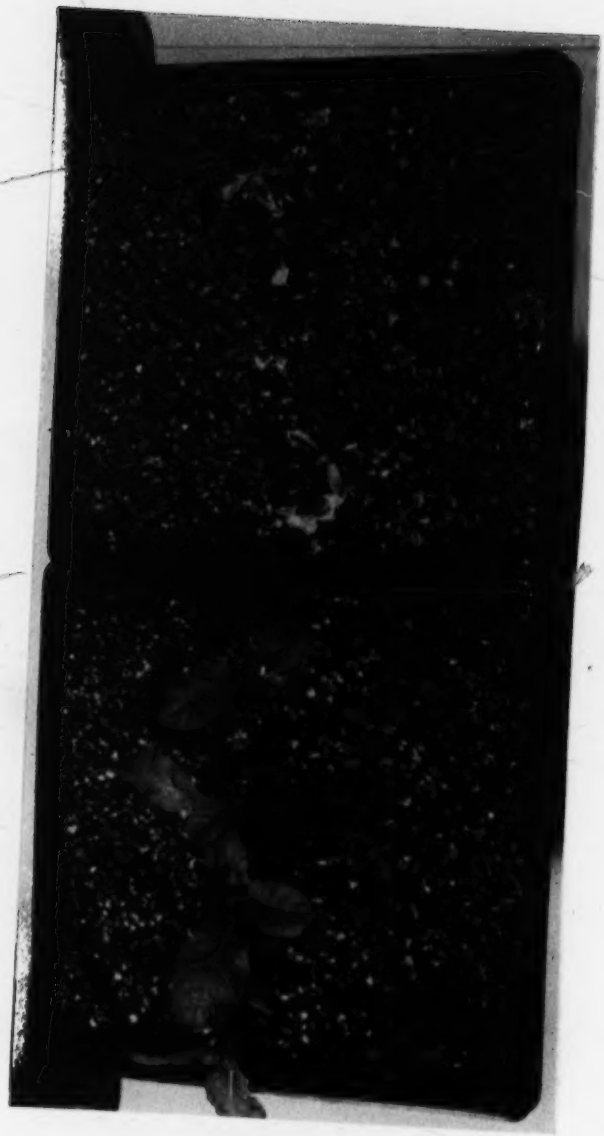

Fig. IV-3. Growth room grown wormseed mustard, peppergrass, and ball mustard (left to right rows) showing the effect of destruxin B . Plants in the top and bottom trays were sprayed with destruxin B and control solutions, respectively. The protocol was identical to that of Fig. IV-1 except that each tray received 3 ml of either solution.

Figs. IV-4 and 5. Growth room grown barley, oats, and wheat (Fig. IV-4), and rye (Fig. IV-5) showing the effect of destruxin B . Left tray in Fig. IV-4 and right tray in Fig. IV-5 were sprayed with control solution and the other 2 trays were sprayed with destruxin B solution. The protocol was identical to that of Fig. IV-1 except that each tray shown in Figs. IV-4 and 5 received 3 and 1 ml of either solution, respectively.

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Fig. IV-6. Field grown canola cv. Westar, wild mustard, wheat, peppergrass, barley, stinkweed, oats, shepherd's purse, and rye (from second left row to right, a guard row of wheat is at the extreme left) showing the effect of destruxin B. Seeds of each species were sown in a 60 cm long row, and the inter-row spacing was approximately 7 cm. Plot was divided breadth-wise into 2 equal halves, one half (upper) was sprayed with the control solution, and the other half (lower) was sprayed with the toxin solution. The control and the toxin solutions were similar to that of Fig. IV-1. Each half received 9 ml of either solution. The reactions of the plants were noted after 6 days of spraying.

Fig. IV-7. A closer view of the field grown canola cv. Westar and wild mustard (2nd and 3rd rows from left, respectively) showing reactions to control solution (upper half) and destruxin B solution (lower half). The protocol was identical to that of Fig. IV-6.

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Fig. IV-8. A closer view of the field grown barley, stinkweed, and oats (left to right) showing reactions to control solution (upper half) and destruxin B solution (lower half). The protocol was identical to that of Fig. IV-6.

Table IV-1. Effect of destruxin B on height, fresh weight, and dry weight of different plant species.

	Pepper-grass		Shepherd's Purse		Stinkweed		Wild Mustard		Barley		Oats		Rye		Wheat		Canola cv. Westar	
	C	Des. B	C	Des. B	C	Des. B	C	Des. B	C	Des. B	C	Des. B	C	Des. B	C	Des. B	C	Des. B
Height (cm)	2.7	1.1*	1.0	0.67	2.4	1.0*	14.5	5.4*	25.8	25.2	24.9	25.6	10.8	11.2	19.5	20.3	13.6	7.5*
Fresh Weight (g)	0.88	0.42*	0.70	0.46	1.62	0.36*	35.28	1.88*	30.45	29.20	21.57	20.08	0.62	0.46	17.85	18.23	26.43	9.81*
Dry Weight (g)	0.11	0.05*	0.12	0.09	0.24	0.11*	4.01	0.36*	3.87	3.65	2.95	2.73	1.52	1.55	2.41	2.44	2.26	0.95*

C and Des. B represents control and destruxin B - sprayed treatments, respectively.
Values are means of four replicates.
* Means significantly different from control of the same species at the 5% level.

D. Discussion

The results of both growth room and field studies showed that destruxin B affected all cruciferous weeds tested except shepherd's purse. The toxin caused severe chlorosis and necrosis in stinkweed and wild mustard, that often resulted in the death of the plants. The height, fresh weight, and dry weight data also supported the above conclusion and suggested that the toxin can be used as a herbicide to control these weeds. The toxin caused severe chlorosis and necrosis in ball mustard, canola cvs. Tobin and Westar, and wormseed mustard. It also caused significant reduction in their heights, fresh weights, and dry weights. As was expected, the host-specific toxin did not affect barley, oats, rye, and wheat. Results considered together suggested that the toxin can be used to effectively control these weeds in cereals.

Toxin-treated peppergrass plants developed moderate levels of chlorosis and necrosis and there was significant reduction in their height, fresh weight, and dry weight compared with that of the control plants. This moderate effect may be due to leaf surface structure which did not collect the toxin droplets or it could be due to the inherent resistance of this plant to the toxin. The results presented in chapter number 2 of this thesis also indicated that *A. brassicae* hosts show a range of sensitivities to the toxin. The response of shepherd's purse plants from the growth room study did not match with their response from the field study. In the growth room, the plants developed severe chlorosis and necrosis that in most cases lead to plant death, but in the field the effect was much less severe. One reason for the less severe effect in the field may be due to seeding late in the season, resulting in small size of plants at the time of spraying.

Synthetic herbicides are available to control cruciferous weeds (Anonymous, 1984). However, they may be sources of environmental pollution, because most of them are halogenated hydrocarbons and are not easily degraded (Duke, 1986b; McWhorter and Chandler, 1982). The toxin produced by *A. brassicae* is a natural compound and should be easily degradable because it contains only carbon, hydrogen, oxygen, and nitrogen.

Almost all host-specific toxins have been discovered because they are involved in diseases of economically important crops (Daly, 1987; Scheffer and Livingston, 1984). Host-specific toxins that recognize only one gene, for example HV-toxin produced by *Helminthosporium victoriae* Meehan & Murphy (Meehan and Murphy, 1947; Scheffer, 1976), are toxic only to the plant containing that particular gene. Therefore, these toxins are useful herbicides only if they work against a pernicious weed. Efforts are being directed towards the discovery of host-specific toxins from weed pathogens (Duke, 1986b). However, so far there is no success in this regard, except for a brief reference of a toxin (bipolaroxin) produced by *Bipolaris cynodontis* (Marig.) Shoem, a fungus infecting a select range of weeds including bermuda grass (Anonymous, 1986b). Nonhost-specific toxins are toxic to a larger number of plants but their use as herbicide is limited because in most cases they are also toxic to economically important plants (Duke, 1986b). However, there are a few exceptions, such as tentoxin which can be used against some weeds in maize (Durbin and Uchytil, 1977). Host-specific destruxin B used as a herbicide combines the useful herbicidal traits of both host- and nonhost-specific toxins. It controls many cruciferous weeds and does not affect cereals, therefore it has the potential of being developed as a broad spectrum herbicide with desirable specificity. The toxin also adds a new chemical class of small cyclic peptides to the herbicide directory, and may help to develop some other herbicides.

Large scale production of the toxin is a prerequisite for its commercial application as herbicide. Total synthesis of destruxin B was described as early as 1965 (Kuyawa and Tamura, 1965). However, recent advances in fermentation technology provide an attractive alternative for large scale production of the toxin. Bialophos, a bioherbicide is currently produced by fermentation from *Streptomyces viridochromogenes* (Krainsky) Waksman and Henrici (Tachibana *et al.*, 1986).

Chlorosis and necrosis caused by destruxin B seem to be restricted around the point of application, thereby, indicating it to be a contact herbicide. Under the conditions of experiments, destruxin B was a very effective herbicide, its efficacy may be further

improved with better formulation and method of application.

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V. General Discussion and Conclusions

A. General Discussion

In earlier studies it was indicated that *Alternaria brassicae* (Berk.) Sacc. produced phytotoxic compounds *in vitro* (Ayer and Peña-Rodriguez; Degenhardt, 1978; Husain and Thakur, 1966). However, except for the concurrent study of Ayer and Peña-Rodriguez (1987), the other two studies were conducted using crude culture filtrates of the fungus or semi-purified toxic fractions from the filtrates. Availability of toxin in the pure form is a prerequisite to establish the toxin as the biochemical basis for disease resistance, disease susceptibility, and host specificity (Scheffer and Livingston, 1984), and also to elucidate its mode of action (Daly, 1981).

The new chromatographic procedure developed to purify *A. brassicae* toxin included gel filtration, adsorption on charcoal, silicic acid chromatography and high pressure liquid chromatography (HPLC). Most purification schemes developed for different toxins include different chromatographic techniques in various combinations, and in many cases HPLC is used as the final purification step. This is due to the excellent resolution and reproducibility of HPLC (Walton and Earle, 1984a). Negative ninhydrin reaction of HPLC-purified *A. brassicae* toxin and the presence of amino acids in the toxin hydrolysate indicated that the toxin is a cyclic peptide. Two other *Alternaria* toxins, AM-toxins I and II produced in culture by *A. alternata* (Fr.) Keissler apple pathotype are also reported to be cyclic depsipeptides (Ueno *et al.*, 1975). Molecular weight, amino acid composition, and ^{1}H NMR spectrum of the toxin were similar to those of destruxin B, a cyclic depsipeptide toxin purified from culture filtrates of *A. brassicae* (Ayer and Peña-Rodriguez, 1987). HPLC retention time of an authentic sample of destruxin B obtained from Dr. W.A. Ayer was also identical to that of the toxin isolated during this study, and a 1:1 mixture of destruxin B and the toxin eluted as a single symmetrical peak. Both destruxin B and the toxin also showed similar biological activity on susceptible (*Brassica campestris* L. var. *yellow sarson*) and resistant (*B. campestris* ssp. *rapifera*) hosts of *A. brassicae*. Because of

the high resolution of HPLC, identical retention times provide *prima facie* evidence that the two compounds are identical (Walton and Earle, 1984a). Based on retention times of HPLC peaks and NMR data, Scheffer and Livingston (1984) also suggested that their HS-toxins A, B, and C produced by *Helminthosporium sacchari* (Van Breda de Haan) Butler are identical to those of Macko *et al.* (1981). Similarly, Clouse *et al.* (1985), based on identical properties relating to NMR spectra, retention time on HPLC, relative mobilities on TLC, and dilution end point of leaf bioassay suggested that both old and the new procedures resulted in purification of the same *A. alternata* f. sp. *lycopersici* toxin (AAL-toxin). This evidence taken together indicated that the toxin isolated during this study is destruxin B. It is a unique phytotoxin because it is also produced by *Metarhyzium anisopliae* (Metschn.) Sorokin, a pathogen of insects (Pais *et al.*, 1981; Tamura *et al.*, 1964), but a non-pathogen of rapeseed (Williams, 1985).

Both *A. brassicae* and destruxin B caused symptoms of different severities on different brassicas, ranging from severe chlorosis and necrosis to almost no visible symptoms. The order of sensitivity of different brassicas to the toxin was similar to the order of susceptibility of these brassicas to *A. brassicae*. The toxin caused no symptoms on plants that are nonhosts of *A. brassicae*. In the present study, sensitivity of a plant to destruxin B was correlated with its susceptibility to *A. brassicae*, suggesting that the activity of the toxin is host-specific and is a factor in disease development. Since host range of *A. brassicae* includes many cruciferous species, and the toxin produced by it has host-specific activity, it follows that the toxin has a wide degree of specificity. Host-specific toxins are known to show various degrees of specificities. *Helminthosporium victoriae* Meehan & Murphy toxin (HV-toxin) has a very high specificity, because it recognizes only one gene (victoria) of oats (Meehan and Murphy, 1947; Scheffer, 1976), whereas *Hypoxylon mammatum* (Wahl.) Miller toxin has a wider specificity that is expressed at the species level and affects the host species *Populus tremuloides* Michx. (Stermer *et al.*, 1984).

Studies on the mechanism of resistance to *A. brassicae* have indicated that both physical and chemical resistance factors are involved. The presence of epicuticular wax

confers a physical type of resistance to *A. brassicae* (Skoropad and Tewari, 1977; Tewari and Skoropad, 1976). The wax appears to confer resistance by creating a hydrophobic surface that decreases retention of *A. brassicae* conidia, and perhaps by restricting the movement of plant exudates reduces both the rate of germination of the conidia and the number of germ tubes per conidium (Conn, 1986). The susceptibility differences of various crucifers to *A. brassicae* may also be due, in part, to qualitative and/or quantitative differences in phytoalexin production (Conn *et al.*, 1988). The results of the present study suggested that the sensitivity of a plant to destruxin B was correlated with its susceptibility to *A. brassicae*, indicating that destruxin B is a factor in disease development.

The minimum concentration of destruxin B (15-30 μg/ml) required for causing visible chlorosis in leaf bioassay is higher than that of the other host-specific toxins. The reason for this high minimum concentration may be the low sensitivity of the scratched leaf bioassay method used in this study. Therefore, it was desirable to develop a more sensitive bioassay method for destruxin B.

Development of a sensitive bioassay technique is a prerequisite for many different aspects of toxin research. Four different bioassay methods were compared for their sensitivity, speed, and technical complexity. The limit of detached leaf bioassay to detect destruxin B was between 15 and 30 μg/ml, and the method could distinguish all two-fold dilutions of the toxin between 15 and 120 μg/ml. It was also sensitive enough to distinguish susceptibility differences among different brassicas and within the species *B. campestris*. The bioassay was easy to perform but took about 48 h to indicate the results. Although the assay was neither very sensitive nor very quantitative, it is still a widely used bioassay method for many toxins (Gardner *et al.*, 1985; Namiki *et al.*, 1986; Vidhyasekaran *et al.*, 1986). In fact, this is generally the first bioassay method used to demonstrate the role of toxin in disease development.

Electrolyte leakage bioassay for destruxin B did not reveal rapid loss of electrolytes. Although the assay could detect 10 μg/ml of destruxin B, it was not quantitative. The assay was simple to perform and took about 48 h to indicate the results. Electrolyte

leakage has proven to be an excellent bioassay technique for many other toxins (Damann *et al.*, 1974; Scheffer and Livingston, 1980; Samaddar and Scheffer, 1971; Tsuge *et al.*, 1985; Yamamoto *et al.*, 1984), but the results of this study have indicated that it is neither sensitive nor quantitative for bioassaying destruxin B.

Leaf mesophyll protoplasts from the susceptible host treated with destruxin B concentration as high as 100 μg/ml showed survival rate similar to that of the control treatment. Although protoplast bioassay has been reported to be a sensitive technique (Walton and Earle, 1984b), the results of this study suggest that it is not a suitable bioassay for destruxin B.

Pollen germination bioassay was the most sensitive, quantitative, fast, and easy to perform bioassay for destruxin B. After only 30 min of incubation, destruxin B at 2.5 μg/ml caused significant inhibition of both germination and tube growth of the susceptible pollen. A complete inhibition of germination was observed at a toxin concentration of 7.5 μg/ml. The pollen germination bioassay is not a widely used. It has been used to bioassay HMT-toxin (Laughnan and Gabay, 1973), and culture filtrates of *A. brassicicola* (Hodgkin and MacDonald, 1986). The results of Hodgkin and MacDonald (1986) also suggested that the bioassay is sensitive, fast, and simple.

Rapid electrolyte leakage has been used to be an indication of initial site of toxin action on the plasma membrane (Kohmoto *et al.*, 1987; Maekawa *et al.*, 1984; Nishimura and Kohmoto, 1983; Scheffer and Livingston, 1984). Absence of rapid electrolyte leakage observed in this study suggests that plasma membrane may not be the initial site of destruxin B action. Delayed electrolyte leakage and increases in the amounts of all metabolites and electrolytes, except sodium, tested in the ambient solutions, collected after 48 h of incubation of susceptible leaf disks with destruxin B, suggested that the toxin eventually caused disruption of the plasma membrane. Delayed electrolyte leakage has also been reported for AAL-toxin, and HC-toxin produced by *H. carbonum* Ullstrup (Kohmoto *et al.*, 1982; McFarland and Gilchrist, 1982; Scheffer and Livingston, 1984; Yoder and Scheffer, 1973). Furthermore, the initial site of AAL-toxin action is not the plasma

membrane, but the enzyme aspartate transcarbamoylase (Gilchrist, 1983; McFarland and Gilchrist, 1981), and as yet there is no proposal for the mechanism of HC-toxin action (Daly, 1981; Scheffer and Livingston, 1984). Nonsensitivity of susceptible protoplasts to destruxin B suggested that the protoplasts may not have the receptor site for the toxin, whereas presence of such a site in the intact leaf tissue and pollen is indicated by their sensitivity to the toxin. Although it is possible that receptor sites may have been stripped away during isolation of protoplasts the results of other studies suggest that this is not so (Briggs *et al.*, 1984; Hawes, 1983). The aforesaid evidence taken together suggested that the cell wall and/or periplasmic space might be the initial site of destruxin B action. Based on ultrastructural effects, the plasma membrane/cell wall domain of the cell has also been suggested to be the initial action site of ACRL-toxins (Kohmoto *et al.*, 1987), AF-toxins I and II, AK-toxin, and AM-toxin I (Park *et al.*, 1981), the toxins produced by rough lemon, strawberry, Japanese pear, and apple pathotypes of *A. alternata*, respectively. However, Kohmoto *et al.* (1987) suggested that physiological and ultrastructural evidences taken together indicate that AF-toxins I and II, AK-toxin, and AM-toxin I have their initial sites of action on the plasma membrane. Studies of ultrastructural effects of destruxin B and specific binding of the toxin to this region may provide further support to the proposed site of destruxin B action.

The results of the pollen germination study indicated that germination and tube growth of pollen from susceptible (*B. campestris* var. *yellow sarson*) plants were completely inhibited after 2 h of incubation with 12.5 μg/ml of destruxin B. In contrast, complete inhibition of both pollen germination and tube elongation from resistant (*Camelina sativa* (L.) Crantz) plants were not achieved even with 100 μg/ml of destruxin B (maximum concentration tested). Differential germination response of pollen from susceptible and resistant plants suggested that *in vitro* culture of microspores in the presence of destruxin B should be an excellent system for *in vitro* selection for *A. brassicae* resistance. Use of toxins for *in vitro* selection for disease resistance has been suggested to be one of the successful strategies (Ingram and MacDonald, 1986).

Weeds cause billions of dollars of damage annually. Biologically produced compounds are becoming increasingly popular than synthetic chemicals to control the weeds. These compounds are easily degradable, and are environmentally safer than synthetic chemicals. Destruxin B has host-specific activity, and host range of *A. brassicae* includes a large number of cultivated and weed plant species belonging to the family Cruciferae (Ellis, 1971). Cereals are known to be nonhosts of the fungus. Based on this information, a practical usage of destruxin B was investigated.

Destruxin B severely affected all cruciferous weeds tested except peppergrass and shepherd's purse. Stinkweed and wild mustard, two serious weeds of cultivated lands (Best and McIntyre, 1975; Mulligan and Bailey, 1975), developed severe chlorosis and necrosis that often resulted in the death of the plants, whereas, plants of barley, oats, rye, and wheat were not affected by the toxin. The results suggested that destruxin B can be used effectively to control these weeds in cereals. Host-specific toxins with extremely high specificity, such as HV-toxin (Meehan and Murphy, 1947; Scheffer, 1976), can only be used as commercial herbicides if they are produced by a pathogen of a pernicious weed. Nonhost-specific toxins on the other hand are toxic to many plants including economically important ones. Therefore, their chances of being used as herbicides are limited. It seems that destruxin B has the desired specificity of a herbicide against weeds belonging to the family Cruciferae. The discovery also adds a new chemical class of small cyclic peptides to the herbicide directory, and may help to develop some other herbicides. Total synthesis of the compound was reported as early as 1965 (Kuyama and Tamara, 1965). However, recent advances in fermentation technology offer an attractive alternative for large scale production of destruxin B. Perhaps, efficacy of destruxin B could be further improved by better formulation and method of application.

B. Conclusions

1. A new chromatographic procedure was developed to purify the toxin produced in culture by *Alternaria brassicae*. Analyses of some chemical and biological characteristics of the purified toxin showed it to be destruxin B, a toxin originally isolated and identified by Dr. W.A. Ayer from *A. brassicae* culture filtrates.
2. It was established that toxin is host-specific and is a factor in disease development.
3. Four bioassay techniques for the toxin were compared. A bioassay involving inhibition of germination and tube growth of susceptible pollen was the most sensitive, fast, and simple to perform.
4. Differential germination response of susceptible and resistant pollen in the presence of destruxin B is likely to form the basis for an excellent system for *in vitro* selection for *A. brassicae* resistance.
5. The results suggest that the target site of the toxin may be in the region of cell wall and/or periplasmic space.
6. The toxin was potentially an effective herbicide against some important cruciferous weeds and volunteer canola in cereal crops.

This study has provided some information that could prove to be useful for future investigations on blackspot of rapeseed. It made some original contributions to the understanding of the mode of pathogenesis in this disease, and proposed a hypothesis for the initial site of destruxin B action. The study suggested a system that can be used for *in vitro* selection for *A. brassicae* resistance. The investigation also described a novel, practical application of destruxin B. This is the first report that describes the usage of a host-specific microbial toxin to control weeds in economically important crops.

C. Suggestions for Future Work on this Topic

1. Time-course ultrastructural studies of toxin-treated susceptible and resistant hosts should be undertaken to confirm the initial site of toxin action.
2. Somaclonal variation provides a novel source of variation for the development of disease resistant crop plants (Ingram and MacDonald, 1986; Larkin and Scowcroft, 1981). Tissue or cell culture in the presence of destruxin B would be an excellent system for *in vitro* selection for *A. brassicae* resistance.
3. Since destruxin B effectively controlled some of the cruciferous weeds under the conditions of this study, efficiency of the toxin to control these weeds under different sets of conditions should be evaluated. Large scale production of the toxin is a prerequisite for it to be used as a herbicide on a commercial basis. Studies should be undertaken to find a cost efficient method for destruxin B production.

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